UNIVERSIDADE FEDERAL DO PAMPA
CAMPUS URUGUAIANA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

TESE DE DOUTORADO

CARACTERIZAÇÃO DAS PROPRIEDADES ANTIOXIDANTES, HIPOGLICEMIANTES E NEUROPROTETORAS DA ERVA MATE EM DIFERENTES MODELOS

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Uruguaiana, RS, Brasil

2017.

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Bioquímica da Universidade Federal do Pampa (UNIPAMPA, RS), como requisito parcial para obtenção do grau de Doutora em Bioquímica.

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Uruguaiana, RS, Brasil
2017

d155c de Lima, Maria Eduarda
CARACTERIZAÇÃO DAS PROPRIEDADES ANTIOXIDANTES, HIPOGLICEMIANTES E NEUROPROTETORAS DA ERVA MATE EM DIFERENTES MODELOS / Maria Eduarda de Lima.
115 p.

Tese(Doutorado)-- Universidade Federal do Pampa, DOUTORADO EM BIOQUÍMICA, 2017.
"Orientação: Vanderlei Folmer".

1. Ilex paraguariensis. 2. Diabetes mellitus. 3. Neuroproteção. 4. Antioxidante. I. Título.

Maria Eduarda de Lima

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elaborada por

Tese apresentada ao Programa de Pós-graduação Stricto Sensu em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Doutora em Bioquímica.

Área de concentração: Bioprospecção Molecular

Tese defendida e aprovada em: 04/08/2017. Banca examinadora:

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Uruguaiana, 04 de Agosto de 2017.

Dedico esta tese à minha amada mãe Iara Lima, que foi mãe e pai em tempo integral e não mediu esforços para que eu chegasse até aqui.

AGRADECIMENTOS

A Universidade Federal do Pampa por tornar esse sonho possível, e principalmente a minha mãe que foi meu alicerce e fonte de amor inesgotável ao longo desta caminhada.

Ao meu orientador, pai científico e amigo Prof. Dr. Vanderlei Folmer que me apoiou, incentivou, orientou e principalmente inspirou ao longo desses anos, permitindo a realização desta tese.

Ao meu co-orientador prof. Dr. Abel Santamaría, pela dedicação, orientação e comprometimento nos dois anos finais do desenvolvimento desta tese, e também a sua esposa Sônia e seus filhos, meus irmão de coração Úrsula e Alonso, que abriram a sua casa para mim no México e fizeram eu me sentir da família, tornando a estadia mais leve e cheia de amor.

Ao meu irmão Fernando Lima, meu sobrinho Lucas Lima, minhas tias, tios, primas e primos que sempre deram apoio da melhor maneira possível tornando o caminho mais leve.

Ao meu padrinho Ancelmo Moraes (*in memorian)* que sei que está muito feliz com essa conquista, e ela é nossa dindo.

Aos meus amigos, tanto brasileiros, quanto mexicanos, que entenderam as minhas ausências e me deram apoio incondicional.

Aos meus colegas do laboratório 403 que foram fundamentais para a realização deste trabalho, principalmente, Mariane, Dérick, Andreia, Hemerson, Aline e Ana.

Aos colegas do laboratório de aminoácidos excitadores, do México, que foram pacientes em ensinar e contribuir para o desenvolvimento desta tese, principalmente Hugo, Eri, Marisol e Gabriela.

A todas as pessoas que direta ou indiretamente contribuíram para a realização deste trabalho.

RESUMO

Tese de doutorado - Programa de Pós-Graduação em Bioquímica
Universidade Federal do Pampa

Caracterização das propriedades antioxidantes, hipoglicemiantes e neuroprotetoras da erva mate em diferentes modelos

Autora: Maria Eduarda de Lima
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Co-orientador: Prof. Dr. Abel Santamaría
Data e local da defesa: Uruguaiana, 04 de Agosto de 2017.

A erva mate é o produto do processamento de folhas secas e moídas de uma espécie arbórea, conhecida como *Ilex paraguariensis*. A apresentação comercial da erva-mate é utilizada no preparo de uma bebida tradicional, muito consumida na América do Sul. Dados da literatura tem comprovado uma diversidade de efeitos benéficos da erva-mate, principalmente o antioxidante. Sabe-se que uma grande quantidade de patologias apresenta o estresse oxidativo como um dos seus mecanismos nocivos, desta maneira, a busca por compostos naturais que possam balancear tais situações foi intensificada nos últimos anos. Considerando os aspectos mencionados, o objetivo principal do presente estudo foi avaliar os possíveis efeitos protetores do extrato de *Ilex paraguariensis* em diferentes perspectivas com ênfase no estresse oxidativo. Inicialmente, foi realizada a caraterização do extrato por HPLC, seguido por uma comparação *in vitro* em sinaptossomas, do extrato (200 mg/mL) com o seu composto majoritário, ácido clorogênico CGA (2 mg/mL), quanto a produção de ROS, peroxidação de lipídios, função mitocondrial por MTT e a razão GSH/GSSG. Por conseguinte, foi realizada a avaliação *in vivo*, onde ratos Wistar machos (300-330 g) foram submetidos a estresse por restrição de movimentos, associado a outros estímulos estressantes, por 21 dias, 6h por dia (crônico) e tratados, por via oral, concomitantemente com extrato (200 mg/mL) ou CGA (2 mg/mL) e foram analisados parâmetros comportamentais de atividade locomotora e ansiedade, bem como, aspectos de dano celular, em córtex, hipocampo e estriado. Por fim, em um modelo de camundongos diabéticos, induzido com estreptozotocina (100 mg/kg), foram verificados os efeitos do extrato de erva mate (850 mg/kg), por via oral, sobre os níveis de glicose, neuropatia diabética e marcadores de estresse oxidativo, em rim, fígado e cérebro. O

extrato de erva mate mostrou-se mais efetivo que o ácido clorogênico na proteção do dano oxidativo em sinaptossomas, reduzindo a formação de ROS, peroxidação de lipídios e depleção de glutationa, fatos que podem explicar o efeito do extrato em prevenir a disfunção mitocondrial avaliada. Da mesma maneira, no modelo de estresse por restrição de movimentos, o extrato de erva mate foi mais efetivo que o ácido clorogênico em prevenir as alterações comportamentais dos animais. O grupo que recebeu o extrato como tratamento, apresentou atividade locomotora igual ou superior a do grupo controle. Os animais tratados com extrato também se mostraram menos ansiosos que os do grupo estressado que não recebeu tratamento. Além disso, o extrato exibiu efeito de proteção do dano celular de córtex, hipocampo e estriado, pois os animais tratados apresentaram um maior número de células viáveis nesses tecidos. Com relação aos efeitos do extrato no modelo induzido de diabetes, verificou-se que a erva mate possui atividade de proteção ao estresse oxidativo envolvido na patogênese dessa patologia, além de reduzir de maneira significativa o nível glicêmico dos animais e apresentar efeito protetor à neuropatia diabética. Com base nos resultados encontrados, é possível dizer que, de modo geral, a erva mate atua na proteção de diferentes aspectos do estresse oxidativo devido ao sinergismo dos seus componentes, tendo em vista que o ácido clorogênico isolado não apresentou efeito protetor igual ou superior ao do extrato. Através do seu efeito antioxidante, apresenta benefícios à redução dos danos causados pela hiperglicemia crônica no diabetes *mellitus* e também, na modulação de efeitos neuroprotetores e função mitocondrial.

PALAVRAS-CHAVE: *Ilex paraguariensis*; Antioxidantes; Estresse oxidativo; Dano celular; Estresse crônico;

ABSTRACT

PhD thesis Degree - Graduate Course in Biochemistry Federal University of Pampa, RS, Brazil

Caracterização das propriedades antioxidantes, hipoglicemiantes e neuroprotetoras da erva mate em diferentes modelos

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Place and date of Defense: Uruguaiana, August 4th 2017.

Yerba mate is the product of the processing of dried and ground leaves of a tree species, known as *Ilex paraguariensis*. The commercial presentation of yerba mate is used in the preparation of a traditional drink, widely consumed in South America. Data from the literature has proven a variety of beneficial effects of yerba mate, especially the antioxidant. It is known that a great number of pathologies present oxidative stress as one of its harmful mechanisms, in this way, the search for natural compounds that can balance such situations has intensified in recent years. Considering the mentioned aspects, the main objective of the present study was evaluate the possible protective effects of *Ilex paraguariensis* extract in different perspectives with emphasis on oxidative stress. Initially, was made the characterization of HPLC extract, followed by an in vitro comparison in synaptosomes, of the extract (200 mg / mL) with its major compound, chlorogenic acid CGA (2 mg / mL) in ROS formation, lipid peroxidation, mitochondrial function by MTT and the GSH / GSSG ratio. Therefore, in vivo evaluation was performed, where male Wistar rats (300-330 g) underwent movement restriction stress associated with other stress stimuli for 21 days, 6 hours per day (chronic) and treated with extract (200 mg / mL) or CGA (2 mg / mL) and behavioral parameters of locomotor activity and anxiety were analyzed, as well as aspects of cellular damage in the cortex, hippocampus and striatum. Finally, in a model of diabetic mice, induced with streptozotocin (100 mg / kg), were verified the effects of yerba mate extract (850 mg / kg) on glucose levels, diabetic neuropathy and markers Of oxidative stress, in kidney, liver and brain. The yerba mate extract was shown to be more effective than chlorogenic acid in the protection of oxidative damage in synaptosomes, by reducing ROS formation, lipid peroxidation and glutathione depletion, which may explain the effect of the extract in preventing mitochondrial

dysfunction evaluated. Likewise, in the model of stress by restriction of movement, the extract of mate grass was more effective than the chlorogenic acid in preventing the behavioral alterations of the animals. The group that received extract as treatment had locomotor activity equal to or greater than the control group. The animals treated with extracts were also less anxious than those of the stressed group that did not receive treatment. In addition, the extract exhibited a protective effect on cell damage in cortex, hippocampal and striatum, as treated animals had a higher number of viable cells in these tissues. Regarding the effects of the extract in the induced model of diabetes, it was verified that the yerba mate has activity of protection to the oxidative stress involved in the pathogenesis of this pathology, in addition to significantly reduce the glycemic level of the animals and present protective effect to diabetic neuropathy. Based on the results found, it is possible to say that, in general, yerba mate acts in the protection of different aspects of oxidative stress due to the synergism of its components, considering that the isolated chlorogenic acid did not present a protective effect equal or greater than of the extract. Through its antioxidant effect, present benefits in reducing the damage caused by chronic hyperglycemia in diabetes mellitus, in the modulation of neuroprotective effects and mitochondrial function.

Key words: *Ilex paraguariensis*; Antioxidants; Oxidative stress; Cell damage; Chronic stress;

Lista de Ilustrações

Lista de Abreviações

ACTH: Hormônio Adrenocorticotrófico

AGEs: Agentes de Glicação Enzimática

ALT: Alanina Aminotransferase

AST: Aspartato Aminotransferase

ATP: adenosina trifosfato

CAT: Catalase

CGA: Ácido clorogênico

DM: Diabetes *mellitus*

DNA: Ácido Desoxirribonucleico

ERs: Espécies Reativas

ETZ: Estreptozotocina

GC: Glicocorticoides

GCR: Receptores de Glicocorticoides

GPx: Glutationa Peroxidase

GSH/GSSG: Razão Glutationa Reduzida/ Glutationa Oxidada

GSH: Glutationa

H_2O_2: Peróxido de Hidrogênio

HPA: Hipotálamo- Hipófise- Adrenal

Ip: *Ilex paraguariensis*

IpE: *Ilex paraguariensis* extract

NAD: Nicotinamida-adenina-dinucleotido

ROS: "*Reactive Oxygen Species"*

SNC: Sistema Nervoso Central

SOD: Superóxido Dismutase

Trx: Tiorredoxina Redutase

SUMÁRIO

APRESENTAÇÃO

No item **INTRODUÇÃO**, consta uma revisão bibliográfica sobre os temas trabalhados nesta tese. A metodologia realizada e os resultados obtidos que fazem parte desta tese estão apresentados sob a forma de artigo e manuscritos científicos, que se encontram nos itens **ARTIGO** e **MANUSCRITOS**, apresentados na sessão de **RESULTADOS**. No mesmo constam as seções: Materiais e Métodos, Resultados, Discussões e Referências Bibliográficas. No item **DISCUSSÃO GERAL**, é apresentado uma discussão do somatório dos resultados presentes na sessões "artigo" e " manuscritos". O item **CONCLUSÕES**, encontrado no final desta tese, apresenta interpretações e comentários gerais sobre os resultados do artigo e manuscritos presentes neste trabalho. Em **PERSPECTIVAS** são apontados possíveis trabalhos futuros para uma continuidade a partir dos resultados da tese. As **REFERÊNCIAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO**, **DISCUSSÃO GERAL**, **PERSPECTIVAS** e **CONCLUSÕES** desta Tese.

1 INTRODUÇÃO

1.1 Estresse Oxidativo

Durante processos fisiológicos ocorre a produção de algumas espécies reativas, essa produção constitui um processo contínuo envolvendo algumas funções biológicas relevantes (Barbosa et al., 2010). Essas espécies podem apresentar-se como radicais livres ou ainda como espécies não radicalares. Radicais livres são definidos como qualquer átomo ou molécula que apresente em seu orbital mais externo um ou mais elétrons desemparelhados, como por exemplo, o radical superóxido, radical hidroxila, óxido nítrico e peroxinitrito (Halliwel, 2001). Já as espécies não radicalares, como os peróxidos, não possuem elétrons desemparelhados, porém desempenham um papel importante no dano oxidativo devido a sua grande instabilidade (Halliwell, 2001; Barbosa et al., 2010).

Durante o metabolismo celular, os radicais livres atuam como mediadores para a transferência de elétrons nas várias reações bioquímicas. Sua produção, em proporções adequadas possibilita a geração de energia na forma de ATP como, por exemplo, por meio da cadeia transportadora de elétrons. Por outro lado, a produção excessiva pode conduzir a danos oxidativos nas biomoléculas (Shami et al., 2004; Barbosa et al., 2010).

A célula desenvolveu mecanismos de defesa antioxidante enzimáticos e não enzimáticos, com objetivo de limitar os níveis intracelulares de tais espécies reativas ou radicais livres e controlar a ocorrência de danos decorrentes (Bianchi,1999; Shami et al.,2004).

Uma situação onde há um desequilíbrio entre a geração moléculas pró oxidantes e o sistema de defesa antioxidante é conhecida como estresse oxidativo. Esse processo conduz à oxidação de biomoléculas com conseqüente perda de suas funções biológicas e/ou desequilíbrio homeostático, cuja manifestação é o dano oxidativo potencial contra células e

tecidos, podendo, se não combatido, levar à morte celular (Halliwell et al., 2004; Barbosa et al., 2010).

A produção de espécies reativas pode levar a danos oxidativos em proteínas, membranas e DNA, como pode ser visto na figura 1 (Schieber & Chandel, 2014. Além disso, ERs podem atuar nas vias de sinalização celular que modulam várias funções (Galley, 2011).

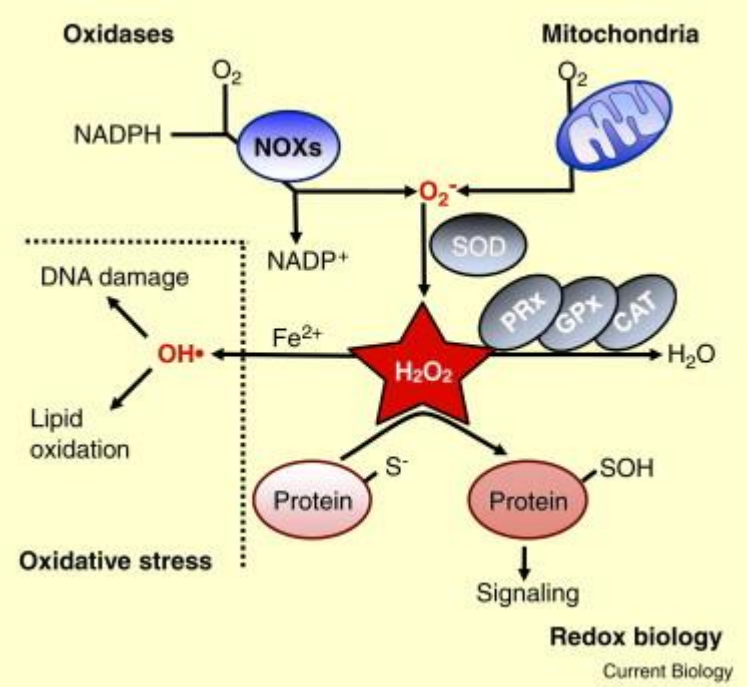

Figura 1 - Papel dos ROS no estresse oxidativo.
Fonte: Schieber & Chandel, 2014.

1.2 Estresse Oxidativo e o *Diabetes mellitus*

Diabetes mellitus é uma desordem metabólica que atualmente é considerada um dos principais problemas de saúde pública em muitos países (IDF, 2015). É caracterizada como um distúrbio endócrino no qual o metabolismo da glicose está alterado. A alteração metabólica pode ser por diferentes maneiras, sendo por uma destruição das células β pancreáticas responsáveis pela produção e liberação de insulina (tipo 1), devido a uma liberação inadequada de insulina pelas células β pancreáticas ou ainda uma insensibilidade à insulina pelos tecidos alvos (tipo 2) (ADA, 2014; Petermann et al., 2015).

Independente da forma de Diabetes, o desenvolvimento patológico de todos tipos leva a uma condição de hiperglicemia. O mecanismo que parece ser comum a todas as células lesadas, como consequência da hiperglicemia, é o estresse oxidativo, com ênfase na produção aumentada de ROS (Brownlee , 2001; Kadenbach et al., 2009; Adly, 2010; Rahal et al., 2014). Este fato coloca a célula em situação de dano oxidativo, quando a condição passa a ser crônica, devido ao desequilíbrio homeostático (Rocha et al., 2006; Reis et al., 2008; ADA, 2014).

A geração aumentada de ROS no diabetes pode ser por uma série de fatores, como a auto-oxidação da glicose, a formação de produtos finais de glicação avançada (AGEs), a via dos polióis e também as mudanças no conteúdo e atividade no sistema de defesas antioxidantes no tecido (Reis, et al., 2008; Silva et al., 2011; Kassab & Piwowar, 2012).

A estreptozotocina é uma glicosamina-nitrosureia comumente usada para produzir o diabetes (Silva et al., 2011). É uma toxina sintetizada pela levedura *Streptomyces achromogenes* (Delfino et al. , 2002). Por possuir uma estrutura similar a molécula de glicose, é facilmente captada pelas células pancreáticas que contêm transportadores de glicose GLUT-2, sendo assim, as células produtoras de insulina que não expressam esse transportador são resistentes à estreptozotocina (ETZ) (Elsner et al., 2000).

Após estar no interior celular, a ETZ desenvolve seus mecanismos de citotoxicidade. O processo é iniciado pela alcalinização do DNA celular e subsequente ativação da poli-ADP ribose sintetase causando depleção rápida e letal de NAD nas células pancreáticas. Posteriormente, ocorre redução no nível de ATP e posterior inibição da síntese e secreção da insulina (Takasu et al., 1999; Bennet et al.,2002). Essas situações culminam com a morte das células β-pancreáticas e consequente incapacidade na produção e liberação do hormônio insulina, o que leva ao desenvolvimento de uma situação de hiperglicemia que ao tornar-se crônica, caracteriza a desordem metabólica Diabetes *mellitus* tipo 1 (Silva et al.,2011). Porém

sabe-se que, além da hiperglicemia, os processos de dano causados pela ETZ também levam a geração de H_2O_2 (Takasu et al., 2001), além da formação e liberação de ROS, através de alguns mecanismos como através da ativação da óxido nítrico sintase endotelial e/ou do sistema Xantina Oxidase (Desco et al., 2002; Matsumoto et al., 2003). Animais tratados com ETZ desenvolvem a maioria das complicações diabéticas mediadas pelo estresse oxidativo tais como a neuropatia diabética (Comelli et al., 2009) e a perda de massa corporal (Wang et al., 2010).

Os efeitos da ETZ estendem-se ainda para além do tecido pancreático (células β-pancreáticas). O dano causado, seja pela droga ou pela consequente hiperglicemia, atingem também o fígado, que é o sítio de metabolização da ETZ e da homeostase de glicose, e também os rins, sítio de excreção da ETZ e do excesso de glicose circulante (Karunanayake et al. , 1976, Maritim et al., 2003).

1.3 Estresse Oxidativo devido a Estresse por Restrição de Movimentos

Uma situação estressante afeta a homeostase corporal, refletindo em alterações bioquímicas e fisiológicas de risco à saúde (McEwen, 2006; Atif et al., 2008).

A imobilização de movimentos tem sido vista como um bom indutor de estresse em animais. Tal protocolo consiste na privação de movimentos espontâneos, conforme pode ser visto na figura 2, induzindo, desta maneira, uma série de alterações físicas e psicológicas nos animais submetidos ao mesmo (Zaid. et al., 2004; Colín-Gonzalez et al., 2015).

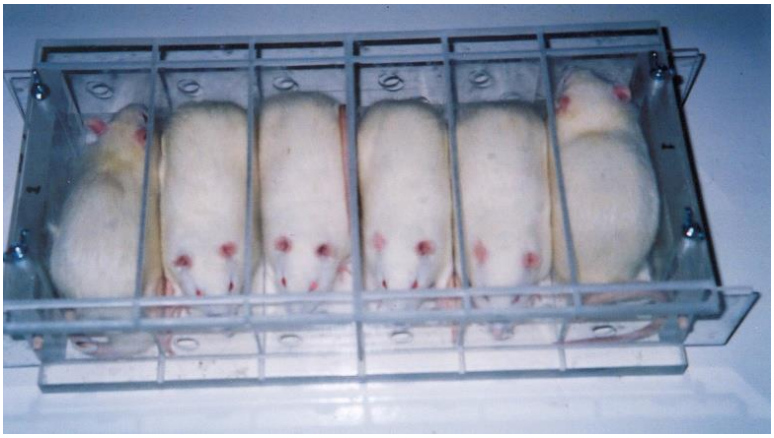

Figura 2 – Dispositivo utilizado no estresse por restrição de movimentos
Fonte: Smith (2012).

Evidências afirmam que o estresse é um dos fatores primordiais para ativar algumas vias celulares que culminam com o aumento na geração de radicais livres (Atif et al., 2008).

O estresse por restrição de movimentos foi relatado por causar um desequilíbrio no estado redox celular, aumentando assim o estresse oxidativo e podendo causar danos oxidativos irreversíveis, como por exemplo, a morte celular (Radak et al., 2001; Colín-González et al., 2015; Becerril-Chávez et al., 2017).

De fato, dados tem demonstrado que animais submetidos a estresse por imobilização apresentam um alto percentual de dano celular em córtex, hipocampo e estriado cerebral (Madrigal et al., 2001; Zaid et al., 2004; Colín-González et al., 2015; Beceril-Chávez, 2017). Além disso, um estudo com ratos mostrou que esse tipo de estresse provoca alterações comportamentais, expressas pelo aumento da atividade ansiolítica e depressão apresentada pelos animais nos testes avaliados (Becerril-Chávez et al., 2017).

Uma das respostas do organismo ao estresse é a ativação do eixo hipotálamo-hipófise-adrenal (HPA), levando à ativação do hormônio liberador de corticotrofina para liberação adicional de hormônio adrenocorticotrófico (ACTH) para a glândula pituitária, gerando a liberação de glicocorticóides (GC) para a glândula adrenal. Esta via é regulada por um feedback negativo induzido pela ligação de GC aos receptores de glicocorticóides (GCR), mantendo-os dentro dos níveis fisiológicos (De Leon et al., 1988; Lucassen et al., 2014).

Vários estudos demonstraram a relação entre o estresse oxidativo, a resposta ao estresse e o desenvolvimento de transtornos psiquiátricos e neurológicos (Sorce, & Krause, 2009; Schiavone et al., 2013), porém o mecanismo exato de indução de dano pelo estresse ao Sistema Nervoso Central ainda está sob investigação. A teoria mais enfática aponta que haja um desequilíbrio entre as defesas antioxidantes e geração de espécies reativas e/ou radicais livres, causando estresse oxidativo.

O cérebro é o órgão mais vulnerável a sofrer efeitos nocivos advindos do estresse oxidativo, pois a sua capacidade antioxidante total é relativamente pequena, além de possuir uma grande quantidade de ácidos graxos poliinsaturados em suas membranas (Halliwell & Gutteridge, 1985; Liu et al. 1994; Zaidi et al., 2004).

1.4 Estresse Oxidativo e os Antioxidantes

De acordo com Sies & Stahl (1995), antioxidante é definido por "qualquer substância que, presente em baixas concentrações quando comparada a do substrato oxidável, atrasa ou inibe a oxidação deste substrato de maneira eficaz". Esses agentes reagem diretamente com as espécies reativas e/ou radicais livres, levando a formação de produtos menos reativos. (Sies, 1993; Halliwell e Gutteridge, 2007).

Em uma situação de desequilíbrio oxidativo os antioxidantes atuam em diferentes níveis na proteção do organismo neutralizando os agentes oxidantes (Halliwell & Gutteridge, 2007; Halliwell, 2011).

O organismo apresenta dois sistemas de defesa antioxidante, o enzimático e o não enzimático, que agem como uma barreira protegendo o corpo dos efeitos nocivos do estresse oxidativo, oque esta expresso graficamente na figura 3.

Figura 3 – Relação de agentes pró-oxidantes e moléculas antioxidantes no estresse oxidativo.
Fonte: Pisoschi & Pop, 2015.

O primeiro mecanismo de defesa endógena é impedir a formação das espécies reativas, principalmente pela inibição das reações em cadeia com o ferro e o cobre. Esses compostos são capazes de agir aos ataques das espécies reativas de oxigênio, impedindo sua formação ou sequestram-nas de forma a não permitir sua interação com alvos celulares (Rover Júnior et al., 2001; Barbosa et al. 2010).

O sistema de defesa enzimático inclui as enzimas Superóxido Dismutase (SOD), Catalase (CAT), Glutationa Peroxidase (GPx) e Tioredoxina redutase (Trx). Essas enzimas agem impedindo e/ou controlando a formação de espécies reativas de oxigênio, convertendo-as em moléculas mais estáveis. A SOD catalisa a dismutação do Ânion Superóxido em Peróxido de Hidrogênio (H_2O_2) e Oxigênio, o peróxido de hidrogênio (H_2O_2), por sua vez pode ser convertido à $2H_2O$ pela Glutationa Peroxidase ou $O_2 + H_2O$ pela Catalase (Ferreira et al., 1997), já as tioredoxinas redutase reduzem tioredoxinas oxidadas (Capacho et al. 2012). O sistema de defesa endógeno não enzimático inclui algumas moléculas, entre elas: a glutationa (GSH), vitaminas C e E (Halliwell & Gutteridge, 2007).

Além disso, há antioxidantes exógenos, principalmente os advindos da dieta, que tem ganho espaço no âmbito da pesquisa (Sies, 1991; Halliwell & Gutteridge, 2007). Alimentos que contêm antioxidantes naturalmente, mas não são ricos em calorias, ou seja, frutas,

legumes e grãos, ajudam a manter a saúde e retardar o início de doenças (Halliwell e Guteridge, 2010; Rahal et al., 2014).

De modo geral, os polifenóis e em particular os flavonóides possuem estrutura ideal para a neutralização de radicais, sendo antioxidantes mais efetivos que as vitaminas C e E (Barreiros et al. 2006; Rahal et al., 2014).

De fato, segundo Samadder et al. (2011) há uma larga utilização de produtos naturais como alternativa terapêutica, no que diz respeito às propriedades antioxidantes da erva-mate, alguns autores sugerem que a ingestão de *Ilex paraguariensis* pode contribuir para aumento das defesas antioxidantes do organismo, minimizando os danos oxidativos associados à formação excessiva de moléculas pró-oxidantes (Schinella et al., 2000; Anesini et al., 2012; Gao et al., 2013).

1.5 *Ilex paraguariensis*

A *Ilex paraguariensis* St. Hil. Var. *paraguariensis* (Aquifoliaceae), conhecida popularmente como erva-mate é uma espécie arbórea nativa da América do Sul e tem sua área de ocorrência natural restrita a três países: Argentina, Brasil e Paraguai (Vieira et al., 2010; Dutra et al., 2010; Júnior & Morand, 2016).

Folhas secas e moídas de *Ilex paraguariensis* são comumente utilizadas no preparo de uma bebida peculiar (Figura 4), consumida por parte da população da América do Sul, que recebe diferentes denominações dependendo de onde é consumida, sendo “chimarrão” no Sul do Brasil, “mate” na Argentina e Uruguai e “tererê” no Paraguai (Heck et al., 2007; Bracesco et al., 2011).

Figura 4 - Folhas secas e moídas de *Ilex paraguariensis* utilizadas no preparo do chimarrão

Além do hábito cultural, a erva-mate também foi muito utilizada na medicina popular para o tratamento de algumas doenças, como a artrite, dor de cabeça, constipação, reumatismo, obesidade, fadiga, hipertensão, desordens hepáticas, entre outros (Pio Corrêa, 1984; Anesini et al. 2006).

Na composição fitoquímica da erva-mate, destacam-se como os principais compostos bioativos os ácidos fenólicos, saponinas e metilxantinas. As propriedades desses compostos estão bem documentadas e alguns dos seus efeitos já foram descritos, como por exemplo, o antioxidante, anti-inflamatório, antiglicação, entre outros (Bracesco et al., 2003; Heck et al., 2007; Filip et al., 2010).

As principais metilxantinas encontradas em extratos de *Ilex paraguariensis* são a teobromina e a cafeína (Colpo et al., 2016; Lima et al., 2017), compostos que exercem ação no Sistema Nervoso Central e que tem sido relatado por serem responsáveis pela ação estimulante exercida pelo chimarrão (Santos et al., 2015).

Por ser tradicionalmente um hábito cultural, a erva-mate passou a ser investigada quanto aos seus possíveis efeitos à saúde. Os primeiros trabalhos realizados publicaram resultados relativos aos seus efeitos antioxidantes *in vivo* e *in vitro* (Gugliucci e Sthal 1995; Campos et al., 1996; Gugliucci 1996). Desde então, uma série de estudos passou a demonstrar uma variedade de efeitos benéficos da erva-mate em diferentes organismos modelos, no qual salientamos nosso grupo, conforme está exposto na tabela 1.

Título	Autor, ano e revista	Resultados principais
Ilex paraguariensis Extract Increases Lifespan and Protects Against the Toxic Effects Caused by Paraquat in *Caenorhabditis elegans*	Lima et al., 2014. International Journal of Environmental Research and Public Health.	Redução do nível de ROS e proteção de efeitos tóxicos do paraquat quanto a sobrevivência e reprodução *in vivo*.
Yerba mate (*Ilex paraguariensis* St. Hill.)-based beverages: How successive extraction influences the extract composition and its capacity to chelate iron and scavenge free radicals	Colpo et al., 2016. Food Chemistry.	Inibição dos radicais livres DPPH e NO. 80% de quelação de ferro *in vitro*.
Protective effect of Yerba mate (*Ilex paraguariensis* St. Hill.) against oxidative damage *in vitro* in rat brain synaptosomal/mitochondrial P2 fractions	Lima et al., 2017. Journal of Functional Foods.	Prevenção da depleção de glutationa e disfunção mitocondrial, associado a redução da formação de ROS *in vitro*.
Compounds from *Ilex paraguariensis* extracts confer antioxidant effects in the brains of rats subjected to chronic immobilization stress	Colpo et al., 2017. Applied Physiology, Nutrition, and Metabolism.	Efeito antioxidante em córtex, hipocampo e estriado *ex vivo* de ratos submetidos a estresse de imobilização.

Tabela 1 - Trabalhos publicados pelo nosso grupo com relação aos efeitos dos extratos de erva mate

O composto majoritário encontrado em alguns extratos de erva mate, por Cromatografia Líquida de Alta Eficiência (HPLC), foi o ácido clorogênico (Bastos et al., 2007; Heck & Mejia, 2007; Menini et al., 2007; De Morais et al., 2009; Filip et al., 2010; Lima et al., 2014; Colpo et. al, 2016; Lima et.al, 2017). Este ácido é um composto fenólico derivado do cafeoilquinico, muito abundante em plantas e bebidas, e tem se destacado por exibir potenciais efeitos biológicos (Kwon et al., 2010; Upadhyay e Mohan Rao, 2013).

Com relação aos efeitos de extratos de erva mate, Schinella et al. (2000) demonstraram que a infusão de erva mate comercial apresentou efeito antioxidante pela inibição enzimática e não enzimática da peroxidação de lipídios.

Ademais, outros resultados publicados a partir de experimentos com infusões de erva mate verificaram alguns efeitos farmacológicos para a espécie: Proteção celular em decorrência do seus efeitos antioxidantes(Bixby et al., 2005); Reversão da resistência à insulina, aumentando a absorção celular de glicose, e a dislipidemia na síndrome metabólica (Hussein et al., 2011); Redução do nível de triglicerídeos, colesterol e redução do índice aterogênico (Balzan et al., 2013); Efeito antiobesidade na reversão da lipogênese hepática induzida por dieta rica em gordura (Resende et al., 2015), entre outros.

A ampla gama de efeitos benéficos reportados, principalmente o antioxidante, tornam os extratos de *Ilex paraguariensis* uma potencial alternativa terapêutica futura no tratamento de desordens que contemplam o estresse oxidativo na sua patogênese.

2 OBJETIVOS

2.1 Objetivo Geral

O objetivo do trabalho é caracterizar as propriedades antioxidantes, hipoglicemiantes e neuroprotetoras da erva mate em diferentes modelos.

2.2 Objetivos específicos

- Investigar *in vivo* os efeitos dos extratos de erva mate sobre as variações da glicemia e sensibilidade térmica como indicador da neuropatia diabética em camundongos com DM induzido por ETZ;

- Quantificar *ex vivo* os efeitos dos extratos sobre a atividade dos marcadores antioxidantes SOD, CAT, ALA-D, TBARS, NPSH e níveis dos marcadores de dano hepático AST e ALT, bem como os níveis de frutosamina de camundongos com DM induzido por ETZ;

- Verificar *in vivo* o efeito dos extratos de erva mate e do ácido clorogênico na atividade locomotora e ansiolítica de ratos submetidos a estrese por restrição crônica;

- Avaliar *ex vivo* o efeitos dos extratos de erva-mate e do ácido clorogênico em aspectos de dano celular no hipocampo, córtex cerebral e corpo estriado destes ratos;
- Comparar os efeitos do extrato de erva mate e do ácido clorogênico em sinaptossomas, quanto à formação de ROS, peroxidação de lipídios e depleção de glutationa;

- Analisar a possibilidade de efeitos protetores desses compostos frente um modelo de disfunção mitocondrial;

3 RESULTADOS

Os resultados da presente tese serão expressos por meio de um artigo científico e dois manuscritos.

Inicialmente, no *Artigo 1*, utilizamos um modelo de sinaptossomas isolados do cérebro de ratos e verificamos aspectos referentes ao estresse oxidativo *in vitro*, onde observamos a formação de ROS, peroxidação de lipídios, depleção de glutationa e função mitocondrial, assim como a possível ação preventiva do extrato em comparação ao CGA nos aspectos avaliados.

No M*anuscrito 1* constam os dados de um modelo de estresse não invasivo, com mecanismo de lesão indireta ao SNC, onde comparamos novamente os efeitos do extrato de erva mate, com o seu composto majoritário, ácido clorogênico (CGA). Os animais foram submetidos a estresse por restrição dos movimentos, associado a outros estímulos estressantes por 21 dias, e foram analisados os efeitos do extrato ou CGA quanto a atividade locomotora e ansiolítica, e também parâmetros de dano celular em córtex, hipocampo e estriado do cérebro destes animais.

Por fim, no *Manuscrito 2*, apresentaremos resultados referentes à avaliação dos efeitos do extrato de erva mate em um modelo de diabetes tipo 1. Neste modelo, camundongos swiss machos, receberam uma dose única de estreptozotocina como indutor de hiperglicemia crônica. Os animais hiperglicêmicos receberam tratamento com extrato de erva mate, ou não (grupo controle) por 90 dias. Posteriormente, analisamos o efeito do tratamento nos níveis de glicose, frutosamina, AST e ALT, no plasma, bem como a neuropatia diabética *in vivo*. Também verificamos *ex vivo* os efeitos do extrato na modulação redox através da atividade e nível de moléculas antioxidantes endógenas.

3.1 Artigo Científico

O seguinte artigo foi publicado em Maio/2017 na revista Journal of Functional Foods , quails A2 na CBII.

Journal of Functional Foods 34 (2017) 447–452

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Short communications

Protective effect of Yerba mate (*Ilex paraguariensis* St. Hill.) against oxidative damage *in vitro* in rat brain synaptosomal/mitochondrial P2 fractions

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ARTICLE INFO

Article history:
Received 6 December 2016
Received in revised form 6 April 2017
Accepted 4 May 2017

Keywords:
Antioxidant defense
Rat brain
Mitochondrial dysfunction
Neuroprotection
Yerba mate extract
Chlorogenic acid

ABSTRACT

Yerba mate extracts are naturally enriched with several antioxidant compounds. Therefore, this natural product constitutes a fertile field of research directed to test its antioxidant and therapeutic properties in the Central Nervous System. We tested the ability of yerba mate to prevent the chemically-induced reactive oxygen species (ROS) formation, lipid peroxidation, glutathione balance (GSH:GSSG) disruption and mitochondrial dysfunction *in vitro* in rat brain synaptosomal/mitochondrial fractions, and compared these effects with those of its most abundant compound chlorogenic acid (CGA). Yerba mate prevented glutathione depletion and mitochondrial dysfunction, and these effects were correlated with its ability to prevent ROS formation. CGA prevented oxidative damage and mitochondrial dysfunction, but its effects were less intense than those of the extract. Our results suggest that the protective properties exhibited by yerba mate cannot be merely attributable to its main component CGA.

1. Introduction

Yerba mate (*Ilex paraguariensis St. Hill*) is a native species from South America with a natural distribution comprising four countries: Brazil, Uruguay, Paraguay and Argentina (Vieira et al., 2010). Dry and crushed leaves of Yerba mate are commonly used to prepare a peculiar beverage which is consumed by the South American population. Among these countries, the beverage receives different names, such as "chimarrão" in south Brazil, "mate" in Argentina and Uruguay, and "tererê" in Paraguay (Bracesco, Sanchez, Contreras, Menini, & Gugliucci, 2011; Heck & Mejia, 2007). Yerba mate-based products are enriched with saponins, alkaloids, flavonoids, vitamins, tannins and polyphenols (Heck & Mejia, 2007). Their biological activities comprise scavenging of reactive oxygen species (ROS) (Dall'Orto, Vago, Carballo, & Rezzano, 2005), interfering with glucose absorption, and modulating the expression of genes and antioxidant enzymes (Bracesco et al., 2011). Methylxanthines and phenolic compounds are essential to confer antioxidant capacity to Yerba mate extracts. The most important property of phenolic compounds is the defense against ROS formation, commonly produced by cell metabolism in response to external factors. In addition, evidence suggests that their actions *in vivo* may involve anti-inflammatory properties (Soto-Vaca, Gutierrez, Losso, Xu, & Finley, 2012) and regulation of energy metabolism (Stevenson & Hurst, 2007).

Chlorogenic acid (CGA) is a polyphenol exhibiting a wide distribution in products used in human diet. CGA has received increasing attention due to its many reported beneficial effects (Heitman & Ingran, 2017). Bracesco and coworkers (2011) have established that CGA corresponds to 42% of the compounds

Abbreviations: 5-CQA, 5-cafeoilquinic acid; CGA, chlorogenic acid; DCF, dichlorofluorescein; DCFH-DA, 2',7'-dichlorofluorescein diacetate; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide; GSSG, oxidized glutathione; GSH, reduced glutathione; LP, lipid peroxidation; MDA, malonaldehyde; 3-NP, 3-nitropropionic acid; OPA, O-phtaldialdehyde; ROS, reactive oxygen species; TBA, thiobarbituric acid; (TBA-RS), thiobarbituric acid-reactive substances.

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http://dx.doi.org/10.1016/j.jff.2017.05.026
1756-4646/

extracted during the consumption of chimarrão, being the majoritarian component of Yerba mate. CGA belongs to a family of esters formed through the interaction of specific trans-cinnamic acids and quinic acid. The commercially available form of CGA is 5-cafeoilquinic acid (5-CQA). Ito, Sun, Watanabe, Okamoto, and Hatano (2008) reported the ability of 5-CQA to cross the blood-brain barrier (BBB), thus reaching the brain. The antioxidant properties of CGA include cardioprotective, antitumor and neuroprotective effects. Its effects at the CNS level are attributable to its ability to react with ROS and chelate toxic metals (Markesbery & Lovell, 2007). These effects include: improvement of spatial learning (Han, Miyamae, Shigemori, & Isoda, 2010), attenuation of memory loss (Kwon, Lee, & Kim, 2010), reduction of anxiety and improvement of motor function (Jang et al., 2013), and enhanced capacity to protect against ischemia-induced neuronal damage (Lee et al., 2012).

Therefore, it is likely that, in addition to the antioxidant properties already reported for Yerba mate, this plant might be a potential source of neuroprotective precursors, including CGA; however, neither its mechanisms, nor the effects of its components have been detailed. In this study, we compared the effects of a typical Yerba mate extract with those of its main component CGA on different oxidative endpoints *in vitro* in rat brain synaptosomal/mitochondrial fractions in order to provide enlightening mechanistic information about the actions and therapeutic potential of this herbal infusion in the CNS. For this purpose, the well-known pro-oxidant iron sulfate ($FeSO_4$) was used to induce oxidative damage through ROS formation, whereas 3-nitropropionic acid was used as an inhibitor of mitochondrial function.

2. Materials and methods

2.1. Chemicals

Yerba mate was purchased in the municipal Uruguayan market of Bella Unión. The aqueous extract was prepared as an infusion of *Ilex paraguariensis* (Aquifoliaceae) at a 200 mg/ml concentration. The infusion was prepared with 10 ml of ultrapure water at 85 °C in 2 g of yerba mate for 10 min. All samples were filtered and diluted 50 times. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), dichlorofluorescein (DCF), O-phtaldialdehyde (OPA), thiobarbituric acid (TBA), HEPES, malondialdehyde (MDA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), M-ethylmaleimide (NEM), sucrose, CGA, and the succinate dehydrogenase inhibitor 3-nitropropionic acid (3-NP), were all obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). CGA was prepared as an original stock containing 20 mg/10 mL of 0.9% saline solution. The final CGA concentration in the 2 mg/mL solution used was 5.6 mM, an equimolar concentration to the CGA contained in the diluted mate extract. This calculation is based on a recent article by our group (Colpo et al., 2016), in which we found that the most diluted extract of the same mate herb used in this study contained 2 mg/mL of CGA, as assessed by chromatographic methods. All other reagents were obtained as reagent-grade from well-known commercial sources.

2.2. Measurement of total content of polyphenols

Measurement of the total content of polyphenols in the mate extract was assayed by a Folin-Ciocalteu's colorimetric method using a mixture of 2% $NaCO_3$ sodium carbonate plus the Folin reagent (1:2). The Mate aqueous extract was prepared as described above, as an infusion (200 mg/mL). The total content of phenolic compounds was evaluated by interpolating absorbance of the samples against the calibration curve constructed with increasing concentrations of gallic acid as standard (10–40 μM), and expressed as μM gallic acid equivalents (GAE). The analyzes were performed in triplicate.

2.3. Chromatographic analyses

The chromatographic system consisted of a Prominence liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a CBM-20A controller, LC-20AT pump, SIL-20A auto sampler, and SPDM20A DAD detector. The chromatographic separation was achieved using an ODS-Hypersil Thermo Scientific C18 column (250 × 4.6 mm i.d., 5 μm particle size) at 25 ± 2 °C. The mobile phase consisted of water containing acetic acid (0.3%, solvent A) and methanol (solvent B). The gradient elution started with 15–20% B for 20 min, followed by 20–85% B for 5 min, which was maintained for 5 min more (Filip, López, Giberti, Coussio, & Ferraro, 2001). The flow rate was 1.0 mL/min and injection volume was 20 μL. Data acquisition by the PDA system was monitored at 265 nm for caffeine and theobromine, and at 325 nm for caffeic and chlorogenic acids. Before analyses, the mobile phase was filtered through a 0.45 μm membrane filter in a solvent filtration apparatus (Millipore, USA), and further sonicated. The reference substances (CGA, caffeic acid, theobromine and caffeine) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and prepared as 50% hydroethanolic solvents. All samples, including extracts, were filtered through 0.45 mm nylon filters and injected into the HPLC. A sample of the cromatographic analysis is shown in Fig. 1.

2.4. Animals

A total of 10 rats were employed throughout the study. Synaptosomal/mitochondrial P2 fractions were collected from the brains of adult male Wistar rats (n = 4 experiments per group) obtained from the vivarium of the 'Instituto Nacional de Neurología y Neurocirugía'. The experiments were strictly carried out following the principles stated in the "Guidelines for the Use of Animals in Neuroscience Research" from the Society of Neuroscience, the local Bioethics Committees, and in compliance of the ARRIVE guidelines.

2.5. Synaptosomes isolation and treatments

Synaptosomal/mitochondrial P2 fractions were obtained from rat brains according to a method reported previously (Rangel-López et al., 2015). Synaptosomes were incubated in a shaking-water bath at 37 °C in the presence of the Yerba mate extract (20 μl of the concentrated solution) or CGA (2 mg/mL = 5.6 mM) for 30 min, and $FeSO_4$ (50 μM) as pro-oxidant or 3-NP (1 mM) as mitochondrial toxin for the next 30 min. $FeSO_4$ was used for the direct induction of oxidative damage through ROS formation, whereas 3-NP was used for the induction of mitochondrial dysfunction through the selective succinate dehydrogenase inhibition. Thirty minutes after incubated, all samples were used for the estimation of all toxic endpoints. The protein content in synaptosomal/mitochondrial fractions was quantified by the Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.6. The assay of lipid peroxidation

Lipid peroxidation was measured in synaptosomal/mitochondrial enriched P2 fractions by TBARS detection, but making modifications, according to a previous report (Colín-González et al., 2015). Two hundred-μL aliquots containing the synaptosomes were incubated with 500 μL of the TBA reagent (0.75 g of TBA + 15 g of trichloroacetic acid + 2.54 mL of HCl) for 30 min at 100 °C. Optical density was measured in a CYT3MV Biotek Cytation 3 Imaging Reader at 532 nm. A standard curve constructed with

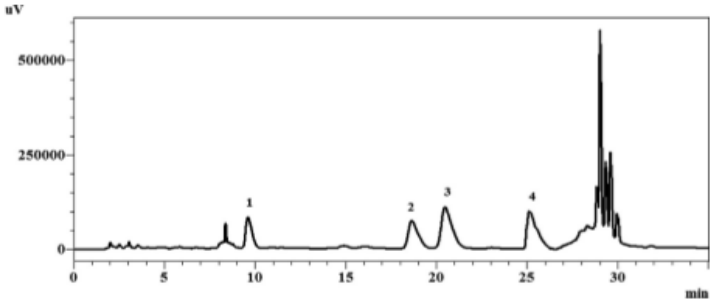

Fig. 1. HPLC analyses obtained from *Ilex paraguariensis* infusion. Peak (1) Theobromine; peak (2) Caffeic acid; peak (3) Chlorogenic acid; peak (4) Caffeine.

tetramethoxypropane was used as an index of the MDA equivalents produced. Results were expressed as nmol of MDA equivalents per mg protein.

2.7. The assay of reactive oxygen species (ROS) formation

ROS production was quantified in synaptosomes according to a previous report (Rangel-López et al., 2015). The synaptosomal/mitochondrial fractions were incubated with 5 μM DCFH-DA for 60 min at 37 °C, and fluorescence was recorded at 488 nm of excitation and 525 nm of emission wavelengths in a CYT3MV Biotek Cytation 3 Imaging Reader. Results were expressed as percent of DCF oxidized vs. control.

2.8. The assay of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction

The MTT reduction assay was used to assess the degree of mitochondrial function, according to a method previously described by Colín-González et al. (2015). The content of formazan was determined in a CYT3MV Biotek Cytation 3 Imaging Reader at a 570 nm wavelength. Results were expressed as percent of MTT reduction compared to control values.

2.9. Estimation of the reduced:oxidized glutathione (GSH:GSSG) ratio

The contents of GSH and GSSG were measured in samples according to methods previously described (Santamaría, Espinoza-González, Ríos, & Santamaría, 1999). For GSH estimation, the synaptosomal/mitochondrial fractions (250 μl) were added to 2.25 ml of phosphate buffer (pH 8.0). Aliquots (50 μl) were added to 50 μl o-phtaldialdehyde (OPA) plus 900 μl phosphate buffer (pH 8.0). Samples were then incubated at room temperature for 15 min, and fluorescence intensity was recorded in a Perkin-Elmer LS55 Luminescence Spectrometer at 420 nm of emission and 350 nm of excitation wavelengths. For GSSG determination, 50 μl aliquots were added to 20 μl N-ethylmaleimide (NEM) to prevent GSH oxidation. Samples were then incubated at room temperature and added to 430 μl 0.1 N NaOH. Fifty μl of this blend were added to 900 μl 0.1 N NaOH plus 50 μl OPA. These samples were incubated once again at room temperature for 15 min and the fluorescence intensity was recorded in a Perkin-Elmer LS55 Luminescence Spectrometer at 420 nm of emission and 350 nm of excitation wavelengths. Results were calculated as nmoles of GSH or GSSG per mg protein. Final results were expressed as the GSH:GSSG ratio.

2.10. Statistical analysis

Mean values ± S.D. were graphically expressed. All data were statistically analyzed by one- or two-way analysis of variance (ANOVA), followed by *post hoc* Bonferroni's test. Values of $p < 0.05$ were considered as statistically significant. For all statistical procedures, the scientific statistic software GraphPad Prism 5 (GraphPad Scientific, San Diego, CA, USA) was used.

3. Results

3.1. Total content of polyphenols

Derived from the values obtained after the calibration curve for gallic acid was carried out, the equation obtained was: $y = 0.0096x - 0.0116$, where 'x' is the concentration of gallic acid, 'y' is the absorbance at 750 nm, and the correlation coefficient is $R = 0.989$. Using these data, we found that GAE concentration in the concentrated extract was 99.125 μM, whereas in the 50-fold diluted sample the GAE concentration was 35.79 μM. These results demonstrated the presence of polyphenols in the extracts used in the study.

3.2. Phytochemical composition

HPLC analysis showed four marker components present in *I. paraguariensis* extract. As shown in Table 1, these phenolics and methylxanthines have been identified as theobromine (9.61 min), caffeic acid (18.71 min), chlorogenic acid (20.54 min) and caffeine (25.39 min) by their retention time and UV absorbance, compared to purified standards. According to the standard peak-area ratio, the relative amounts for each compound detected in Yerba extract presented the following order: chlorogenic acid > caffeine > caffeic acid > theobromine, respectively. Accordingly, a previous study of our group (Colpo et al., 2016) showed that among these compounds, CGA was found in larger amount in yerba mate samples from Uruguay, which motivated us to use this herbal product for present research.

3.3. Yerba mate extract decreased the $FeSO_4$-induced oxidative stress and the 3-NP-induced mitochondrial dysfunction in synaptosomal/mitochondrial P2 fractions

Fig. 2 depicts results of the effects of yerba mate extract on ROS formation (in A), lipid peroxidation (in B) and mitochondrial dysfunction (in C) in rat brain synaptosomal fractions.

Table 1
Phytoconstituents identified in *Ilex paraguariensis* extract.

Compound	mg/mL[a]	UV_{max}	*Rt* (min)
Chlorogenic acid	1.983 ± 0.048	325	20.54
Caffeine	1.655 ± 0.059	270	25.39
Caffeic acid	1.160 ± 0.072	325	18.71
Theobromine	0.476 ± 0.016	270	09.61

[a] Results are expressed as mean values of mg/mL contained in the *I. paraguariensis* extract ± standard deviation. These values represent the average of three analyses.

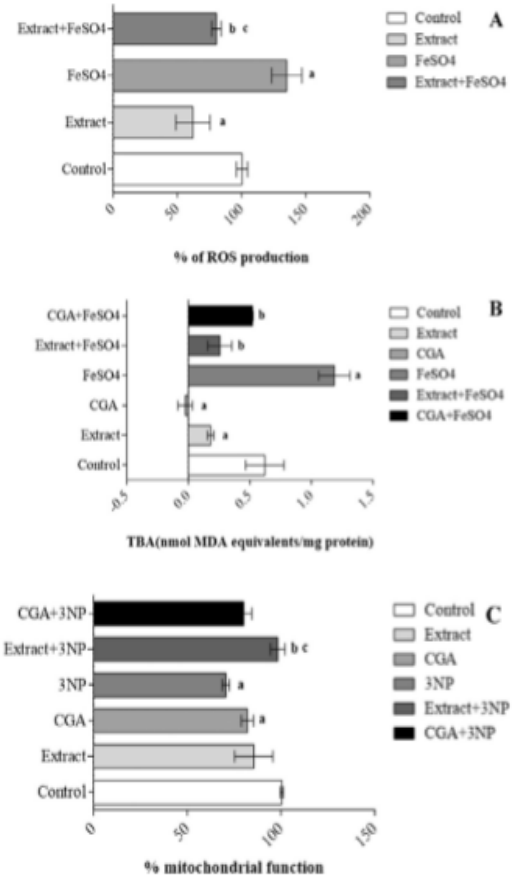

Fig. 2. Effects of Yerba mate extract (200 mg/ml) on $FeSO_4$ (50 μM)-induced oxidative damage, and 3-nitropropionic acid (3-NP; 1 mM)-induced mitochondrial dysfunction in rat brain synaptosomal P2 fractions. In (A) Yerba mate extract was tested against the $FeSO_4$-induced reactive oxygen species (ROS) formation (oxidation of DCFH); in (B) the effect of Yerba mate extract on the $FeSO_4$-induced lipid peroxidation (TBARS formation) was compared with the effect of its main component chlorogenic acid (CGA; 2 mg/mL); in (C) the effect of Yerba mate extract on the 3-NP-induced changes in mitochondrial function (MTT reduction) was contrasted with the effect of CGA. Data are expressed as mean values ± S.D. of n = 4 experiments per group. Symbols denote statistical differences vs. control ($^a p < 0.05$), and against $FeSO_4$ or 3-NP ($^b p < 0.05$). One- and two-way ANOVA followed by Bonferroni's test.

In Fig. 2A, the ROS formation induced by $FeSO_4$ (35% above the control; $p < 0.05$) was prevented by the extract (41% below $FeSO_4$; $p < 0.05$), whereas when tested alone the extract decreased the ROS levels even below the control (40%; $p < 0.05$).

In Fig. 2B, the $FeSO_4$-induced increased lipid peroxidation (88% above the control; $p < 0.05$) was completely prevented by the Yerba mate extract (75% below $FeSO_4$; $p < 0.05$) and by CGA (59% below $FeSO_4$; $p < 0.05$). The extract and CGA *per se* decreased the levels of lipid peroxidation even below the control values (69% and 99%, respectively; $p < 0.05$).

In Fig. 2C, the 3-NP-induced decreased MTT reduction (27% below the control; $p < 0.05$) was prevented by the yerba mate extract (34% above 3-NP; $p < 0.05$). A moderate effect was produced by CGA (8% above 3-NP; N.S.). Both the extract and CGA decreased mitochondrial activity (18% and 20% below the control), but only CGA produced a significant effect.

3.4. Yerba mate extract prevented the disruption of the GSH:GSSG ratio in synaptosomal/mitochondrial P2 fractions

The GSH:GSSG ratio in brain synaptosomes incubated with the Yerba mate extract or CGA, and exposed to $FeSO_4$, was determined after calculating the GSH and GSSG contents. Basal levels of GSH and GSSG in controls were 91.2 and 5.1 nmol/mg protein, respectively. The exposure of synaptosomes to $FeSO_4$ decreased the GSH:GSSG ratio by 74% compared to control ($p < 0.05$), whereas the incubation of these fractions in the presence of the extract or CGA prevented the effect of $FeSO_4$ by 253% above ($p < 0.01$) and 109% above ($p < 0.05$) above the pro-oxidant, respectively (7 and 45% below control, respectively) (see Table 2). The extract and CGA treatments alone did not modify the basal GSH:GSSG ratio.

4. Discussion

In this study, the use of the pro-oxidant agent $FeSO_4$ produced the generation of a model of oxidative damage in synaptosomes through ROS formation. It is known that iron promotes the intense generation of hydroxyl radicals through the Fenton reaction (Yang, Campbell, & Bondy, 2000). Considering the effects obtained in this study with the Yerba mate extract on the prevention of ROS formation at both basal (control) and iron-stimulated levels, it is reasonable to suggest that the different antioxidant components present in the extract (CGA, caffeine, caffeic acid and theobromine) could act coordinately as ROS scavengers. Therefore, these components could trap the ROS generated by Fenton reaction, thus decreasing the potential risk of further oxidative damage in the synaptic terminals. This effect is supported by previous reports demonstrating the antioxidant capacity of the extract under *in vitro* conditions (Colpo et al., 2016; De Oliveira, Calado, Ares, & Granato, 2015).

In *in vitro* preparations of the CNS (brain slices), events such as oxidative damage to proteins are linked with the generation of hydroxyl radicals through the iron-catalyzed Fenton reaction, also compromising the activity of the mitochondrial electron transport chain (Bizzozero, Ziegler, De Jesus, & Bolognani, 2006). It has been demonstrated that the Fenton reaction, and the consequent oxidative activity produced, are capable of inducing the oxidation of P2 synaptosomal/mitochondrial fractions (Yang et al., 2000). Therefore, the oxidative model employed in this study using P2 synaptosomal/mitochondrial fractions supports the concept of ROS generation (mainly hydroxyl radical) by iron-catalyzed reactions, suggesting that the several antioxidant compounds present in the Yerba mate extract could target this catalytic process. It is also well-known that the Fe^{2+}/Fe^{3+} ratio stimulates lipid peroxidation in synaptosomes (Guo, Zhao, Li, Shen, & Xin, 1996). Thus, iron plays a central role in ROS generation because in Fenton reaction H_2O_2 oxidizes Fe^{2+} to Fe^{3+} to generate hydroxyl radicals (Halliwell & Gutteridge, 1992). Moreover, iron initiates a chain reaction leading to lipid peroxidation and the consequent cellular damage. Lipid peroxidation is damaging because the formation of peroxidation products spread further free radical reactions (El-Aal, 2012, chap. 3).

Table 2
Effects of Yerba mate extract (YME) and chlorogenic acid (CGA) on the GSH:GSSG ratio in rat brain synaptosomes.

	GSH/GSSG
Control	17.9 ± 4.8
$FeSO_4$	4.7 ± 7.2[a]
YME	18.5 ± 3.0
CGA	15.9 ± 8.2
YME + $FeSO_4$	16.6 ± 6.4[c]
CGA + $FeSO_4$	9.8 ± 8.4[b]

Mean values ± S.D. of n = 4 experiments per group. Two-way ANOVA followed by Bonferroni's test.
[a] $p < 0.05$, different of control.
[b] $p < 0.05$, different of $FeSO_4$.
[c] $p < 0.01$, different of $FeSO_4$.

Also in this study, it was shown that Yerba mate extracts reduced the TBARS formation induced by Fe^{2+} in rat brain synaptosomes. CGA, the major compound in yerba mate extract (Colpo et al., 2016), also demonstrated a significant capacity to reduce lipid peroxidation in isolated synaptic terminals when administered at an equal molar base than the CGA contained in the Mate extract. In addition to the properties of the extract components as ROS scavengers, these effects may be associated to the iron chelating activity of Yerba mate. It is possible that the extract compounds bind iron coordinately, thus forming a redox complex which reduces the Fe^{2+} availability to interact with H_2O_2 and decrease hydroxyl radical formation, therefore protecting against peroxidative damage. These considerations are reinforced by the complete prevention of the FeSO4-induced GSH:GSSG balance disruption when incubated in the presence of the Yerba mate extract, vs. a partial recovery of this ratio when synaptosomes were incubated in the presence of CGA.

Some studies using natural products, or compounds synthesized from these products, have demonstrated the ability to prevent and sometimes reverse synaptosomal disruption and/or oxidative damage. For instance, the saponin from the *Solanum anguivi* showed an intense capacity to reduce lipid peroxidation induced by Fe^{2+} in the P2 synaptosomal fractions of the rat brain (Elekofehinti et al., 2015), and these properties could account for neuroprotection. Elinos-Calderón et al. (2010) tested the effect of S-allylcysteine (SAC) in synaptosomes submitted to toxic insults, and demonstrated that this garlic-derived compound is able to evoke protective effects even when toxic events in the brain have already started. According to these reports, we propose that the same mechanism described above is responsible for the results of this study: protection against oxidative damage.

On the other hand, 3-NP has been widely used as toxic model as it is an irreversible inhibitor of succinate dehydrogenase (complex II) of the electron transport chain (Ramachandran & Thangarajan, 2016). Such inhibition leads to blocking the transfer of electrons to coenzyme Q, thereby affecting the sequence of events of the respiratory chain, and increasing ROS generation (Ramachandran & Thangarajan, 2016). As a result of this oxidative imbalance, a succession of damaging effects occurs in mitochondria, leading to energy depletion and dysfunction. Indeed, the primary event after succinate dehydrogenase (SDH) inhibition is ROS generation, which subsequently leads to a decreased mitochondrial function and increased lipid peroxidation (Colle et al., 2013).

In this study, we also collected experimental evidence showing that the reduction in mitochondrial function caused by 3-NP can be completely prevented by yerba mate extract, but not by its major compound, CGA. We therefore hypothesize that there is also a synergistic effect of the various compounds present in yerba mate extract, acting together to neutralize ROS generated after blocking SDH, thus avoiding subsequent damage. In support to our observations, a recent report showed that succinobucol, a phenolic compound known for its antioxidant and anti-inflammatory properties, exhibited similar effects to Yerba mate. The mentioned report attributed this effect to the scavenging activity of the compound, which is able to prevent secondary toxic events by neutralization ROS generated after SDH inhibition (Colle et al., 2013).

5. Concluding remarks

Several studies have shown the antioxidant properties of Yerba mate liked to the action of its phenolic compounds, saponins, metilxantines, etc. (Bracesco et al., 2011; Colpo et al., 2016; Heck & Mejia, 2007). Noteworthy, during the use of natural extracts for human consumption, the antioxidant and protective effects obtained could correspond to a given component of the extract or to a synergistic action of its various components. The extract tested in this study has CGA as the main component, a phenolic molecule also known for its antioxidant properties (Feng et al., 2016). However, the results presented here comparing the effects of Yerba mate vs. CGA at an equal molar base, demonstrate that the actions of the latter are limited when compared with the extract. We therefore suggest that there is a coordinated action of the different compounds present in the extract that attenuates oxidative damage and mitochondrial dysfunction in nerve cell fractions. Since the antioxidant effects of Yerba mate cannot be merely attributed to CGA, the medicinal and therapeutic properties of this herb deserve the consideration of all its components.

Competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

Acknowledgements

This work was supported by CONACyT (Mexico) Grant 205648 (A.S.) and CAPES (Brazil) Grants (99999.006894/2015-01 for M.E. L. and 99999.006340/2015-06 for A.C.C.).

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3.2 Manuscrito científico 1

Manuscrito submetido à:

Journal of functional foods, quails A2 na CBII.

Elsevier Editorial System(tm) for Journal of Functional Foods

Manuscript Draft

Manuscript Number:

Title: Comparing the effects of Ilex paraguariensis extracts and chlorogenic acid on behavioral and morphological alterations in rats subjected to chronic restraint stress

Article Type: Full Length Article

Keywords: Brain regions; Oxidative stress; Brain damage; Cell protection; Antioxidant defense; Natural antioxidant compounds

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Abstract: The effects of Yerba mate (Ilex paraguariensis) extracts (IpE) and its main compound chlorogenic acid (CGA) were evaluated on behavioral and morphological endpoints of brain damage induced by chronic restraint stress (CRS) to rats. CRS sessions were performed during 21 days. IpE (200 mg/mL, p.o.) or CGA (2 mg/mL, p.o.) were administered daily 30 min before stress. Behavioral tests comprised motor and anxiety-like activity. Histological changes and the percent of cell damage were estimated in three brain regions: cortex (Cx), hippocampus (Hp) and striatum (S). Rats subjected to CRS exhibited lower locomotor activity. Rats receiving IpE before CRS preserved the basal locomotor activity. Stressed animals augmented the anxiety-like activity, whereas IpE normalized exploratory behavior. Stressed animals presented cell damage in all regions. Morphological damage was more effectively prevented by IpE. Our findings support that the protective effect of IpE against CRS may be related to its antioxidant properties.

Original research

Comparing the effects of *Ilex paraguariensis* extracts and chlorogenic acid on behavioral and morphological alterations in rats subjected to chronic restraint stress

Running head: **Yerba mate extracts and restraint stress**

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ABATRACT

Restraint stress can produce severe damage to brain. The extracts obtained from Yerba mate (*Ilex paraguariensis*) are enriched with natural antioxidants with neuroprotective properties, such as chlorogenic acid (CGA). In this work we evaluated the effects of *Ilex paraguariensis* extracts (IpE), and compared them with those of CGA, on behavioral and morphological indicators of toxicity and brain damage induced by chronic restraint stress (CRS) to rats. CRS sessions were performed every day for 6 h during 21 consecutive days. IpE (200 mg/mL, p.o.) or CGA (2 mg/mL, p.o.) were administered daily 30 min before every single stress session. Behavioral tests comprised two different aspects: motor and anxiolytic-like activity (open field and elevated plus-maze tests, respectively). Histological changes were assessed by Hematoxylin & Eosin staining, and cell damage was estimated in three different brain regions: striatum (S), hippocampus (Hp) and cortex (Cx). Compared to control (unstressed) rats, animals subjected to CRS exhibited a lower locomotor activity. Rats receiving the herb extract before CRS maintained locomotor activity values similar to or greater than the unstressed control group. Stressed animals also augmented the time remaining in close arms of the elevate plus-maze, whereas rats receiving the extract showed a normal exploratory behavior. In contrast, animals subjected to CRS and treated with CGA showed no improvement compared to the CRS group in both tests. Stressed animals also presented cellular damage in all regions analyzed, compared to the unstressed group. Morphological damage was prevented by IpE, but not by CGA. Our findings support the concept that the protective effects exerted by IpE in this toxic paradigm are likely to be related to its broad range of antioxidant properties.

Keywords: Brain regions; Oxidative stress; Brain damage; Cell protection; Antioxidant defense; Natural antioxidant compounds.

1. Introduction

The daily routine favors exposure to different stress conditions. This stress can be mostly acute, which is characterized by a physiological and momentary reaction to a dangerous situation. However, when the stressing conditions are not reversed, stress becomes chronic. Stress causes a number of changes known as the general adaptation syndrome. This is characterized by three phases: alarm, resistance and exhaustion (Selye, 1936; Selye, 1998; Schwarzer, 2001; McEwen, 2006). In the alarm phase, there is an activation of the neuroendocrine system, which is characteristic of adaptive responses to emergency situations. In the resistance phase, the body adapts to the stressor, whereas the exhaustion phase reflects the moment when the adaptation phase of the organism is exhausted or becomes harmful to the body, which can lead to anxiety, depression and even death. The brain is regulator of the neuroendocrine, autonomic, and immune systems, and chronic stress causes changes in brain function that can be seen both in humans and animals (Selye, 1998; McEwen, 2006; Schiavone 2013; Becerril-Chávez, 2017).

The need to model chronic stress in humans has led to the development of animals models. Chronic restraint stress (CRS) is indicated as a good stress inducing model, where the animals remain several hours immobilized and deprived of water and food. Prolonged immobilization leads to the occurrence of biochemical and behavioral alterations in animals, similar to those observed in chronic human pathologies presenting stress as a component of their physiopathology (Glavin, et al., 1994; Liu et al., 2013; Colín-González et al., 2015).

Some studies have demonstrated that animals submitted to stress present a high percentage of cellular damage in the cortex hippocampus and striatum regions. In addition, they show altered behavior related to changes in anxiety-like locomotor

activity and depression (Madrigal et al., 2003; Zaidi & Banu, 2004; Colín-González et al., 2015; Becerril-Chávez, 2017).

The mechanism of induction of stress-related damage to the CNS is still under debate, but it is believed that there is an imbalance between the antioxidant defenses and the generation of reactive species and/or free radicals causing oxidative damage. The brain is the main organ undergoing oxidative damage, as it has low levels of antioxidants and a high content of polyunsaturated fatty acids in its membranes, becoming more vulnerable to the attack of reactive oxygen species (ROS) (Halliwell & Gutteridge, 1985; Floyd, 1991; Liu et al. 1994; Liu et al., 1996; Zaidi & Banu, 2004). In this regard, the daily intake of antioxidants that cross the blood brain barrier - and therefore can exert effects at the level of the CNS - is fundamental to achieve protection against the possible stress affecting body homeostasis (Zaidi & Banu, 2004; Colín-González et al., 2015; Becerril-Chávez et al., 2017).

Ilex paraguariensis is a tree-species native plant of the South-American region used to prepare a cultural drink mainly consumed in Brazil, Argentina, Uruguay and Paraguay. Extracts of *Ilex paraguariensis* are composed of a large amount of phenolic compounds, which are known to have antioxidant action (Lima et al., 2014; Colpo et al., 2016; Lima et al., 2017).

The major component of our *Ilex paraguariensis* extract is chlorogenic acid (Lima et al., 2017). This phenolic compound is capable of crossing the blood-brain barrier in its intact form, or as a metabolite, and may therefore exert antioxidant and neuroprotective effects (Ito et al., 2008; Kwon et al., 2010). The present study aimed to compare the effects of *Ilex paraguariensis* extracts (IpE), and its major compound chlorogenic acid (CGA), on parameters of cellular damage, locomotor activity and anxiolytic activity in different brain regions in a CRS model in rats.

2. Materials and methods

2.1. Chemicals and extracts preparation

The commercial presentation of yerba mate was acquired in a supermarket at the municipal Uruguayan market of Bella Unión. The aqueous extract (200 mg/mL) was prepared as an infusion of *Ilex paraguariensis* (*Aquifoliaceae*). The infusion was prepared with 2 g of yerba mate in 10 ml of ultrapure water at 85 °C for 10 min. All samples were filtered. CGA was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), and prepared as an original stock containing 20 mg/10 mL of 0.9% saline solution. The final CGA concentration in the 2 mg/mL solution used was 5.6 mM, an equimolar concentration to the CGA contained in the mate extract. This calculation is based on a recent article of our group (Lima et al., 2017) in which we found that the most diluted extract of the same mate herb used in this study contained approximately 2 mg/mL of CGA, as assessed by chromatographic methods. All other reagents were obtained as reagent-grade from well-known commercial sources.

2.2. Animals

All animals (N = 54) were obtained from the vivarium of the National Institute of Neurology and Neurosurgery (Mexico). Male Wistar adult rats (300-330 g) were employed throughout the study. Initially, animals were housed in polycarbonate cages (6 per cage) and had free access to food (rodent Chow 5001; PMI Feeds Inc., Richmond, IN, U.S.A.) and water. Housing facilities included controlled conditions of temperature (25 ± 3 ° C), humidity (50 ± 10%) and light/dark cycles (12:12 h).

2.3. Restraint stress protocol and drugs administration protocol

Six experimental groups (n = 6 rats per group, randomly selected) were designed: control (C), *Ilex paraguariensis* extract (IpE), chlorogenic acid (CGA), stress (S), *Ilex paraguariensis* extract + stress (IpE + S), and CGA + stress (CGA+S). All groups under

stress were submitted to immobilization every day during 6 h (from 8:00 am to 2:00 pm) for 21 consecutive days in a glass device with the following dimensions: 6 x 7 x 18 cm. After four hours of stress were completed, the animals were submitted to different types of stimuli during the same 21 days. This protocol was performed to avoid the animals to present adaptation to the restriction stress. The types of stimuli applied were: darkness, inclination of the box, water in the box, application of heat, intense light directed to the box, and ice; all were used alternately during the 21 days of assay. C, IpE and CGA groups were isolated daily in acrylic cages (30 x 30 x 20 cm) during 6 h for 21 days, allowing them freely moving. The six groups were deprived of food and water during the 6 h of isolation. IpE and IpE + S groups also received IpE (200 mg/kg) 30 min before restraint or isolation for the same 21 days, whereas CGA and CGA + S groups received CGA (2 mg/mL). All other groups received water. All groups were administered by gavage. At the end of the chronic stress procedure (21 days), two different behavioral parameters were estimated (elevated plus maze and open field), immediately followed by animal perfusion and euthanasia by decapitation to obtain the brains for the histological assessment by Hematoxylin & Eosin (H&E) staining.

2.4. Elevated plus maze

The elevated plus maze test is commonly used to estimate anxiety-like behavior in rodents. This device consists of a platform in the shape of a plus sign (+), with two opposing arms open and the two closed arms along the sides. This platform is placed at a height of 0.5 m from the ground. Rats were placed in the center of the cross, and their willingness to enter the open arms against the relatively safer closed arms is then assessed. Spontaneously, rodents display exploratory behavior in new environments; however, when animals are anxious, they lose this ability. The parameters estimated during this 5-min test were the number of entries that each animal made to close and

open arms, and the time spent in both type of arms. For all animals, this test was performed 30 min after the last period of immobilization or isolation.

2.5. Open field test

The open field test was carried out immediately after the elevated plus maze test, using an AccuScan device (AccusScan Instruments Inc., Columbus, OH) in a room with controlled conditions of light and temperature, and free of noise. The equipment employs software programmed to detect the number of animal passages over infrared lights on horizontal and vertical axes. The rats were placed in the center of the device and their behavior was recorded for a total period of 15 minutes. All animals were subjected to the open field test only once in order to avoid habituation. The estimated parameters of motor activity included the total vertical and horizontal distances in movement, as well as the number of total movements. The apparatus was cleaned with 70% ethanol before testing each animal.

2.6. Histological assessment

The brains of rats from all experimental groups were perfused transcardially, collected and paraffin embedded to obtain sagittal cuts (7 μm) by a Leica RM2255 microtome (Leica Geosystems AG, St. Gallen, Switzerland). All cuts were fixed, deparaffined and dehydrated to be stained with Hematoxylin & Eosin for the histological analysis of the striatum, hippocampus and cortex. Digital images were obtained by a Nikon ECLIPSE E200 microscope (DiaMedical USA, West Bloomfield, MI), using the Q-capture Pro 7 software. The percent of cell damage was estimated by counting the number of pyknotic cells compared to the total number of cells per field in three fields from three different animals per group.

2.7. Statistical analysis

All data were expressed as mean values ± S.E.M. of 6 rats per group. Results were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons, using Prisma 5 software (GraphPad, San Diego, CA, U.S.A.). Values of $p<0.05$ were considered as statistically significant.

3. Results

3.1. Body weight changes after chronic stress in rats

The body weight of each rat was recorded weekly during the whole experimental protocol (21 days) to monitor the effect of stress. There was a significant reduction in the body weight of animals in the stress group (43 % below control, $p<0.05$), which was prevented in the group of stressed animals also receiving the herb extract (15 % below control, $p<0.05$), and partially prevented by treatment with CGA (33 % below control, $p<0.05$). Figure 1 shows the body weight delta (final weight - initial weight) of all experimental groups.

3.2. Ilex paraguariensis extract reduced the anxiety-like behavior in chronically-stressed rats

To evaluate if CRS induced an anxiety-like behavior in animals, we used the elevated plus-maze test. The number of entries that each animal made to close and open arms, as well as the time spent in both type of arms, were considered. The stress condition produced a reduction in the number of animal visits to close the arms (38 % below control, $p<0.05$) (Figure 2A). The number of entries in the closed arms was lower because the animals remained longer time in the closed arms compared to the control group (36 % above control, $p<0.05$) (Figure 2B), which maintained its exploratory character, passing through the four arms of the cross. The administration of IpE to stressed rats produced a significant decrease in the anxiety behavior (34% below CRS, $p<0.05$), similar to the control group. In contrast, the animals treated with CGA alone were similar to the stressed animals, though stressed animals treated with CGA showed a behavior similar to the control group.

3.3. Ilex paraguariensis prevented motor alterations in stressed rats

Stressed animals presented a lower locomotor activity compared to the control group, suggesting that stressed animals lost their natural exploratory activity as they presented a shorter distance traveled both at vertical (28 % below control, $p<0.05$) (Figure 3A) and horizontal (20 % below control, $p<0.05$) (Figure B) levels, as well as a decreased number of total movements (24 % below control, $p<0.05$) (Figure 3C). The stressed animals treated with IpE showed an increased locomotion activity (12% above control, $p<0.05$) compared to the control group, in contrast to the group treated with CGA.

3.4. Ilex paraguariensis extract attenuated the chronic stress-induced cell damage in cortex, striatum and hippocampus of the rat brain

CRS caused an increase in the ratio of cell damage cells in all regions from the experimental groups, being significant for cortex (310 % above control, $p<0.05$), hippocampus (325 % above control, $p<0.05$) and striatum (225 % above control, $p<0.05$), compared to control. IpE was able to prevent this effect as rats showed less cell damage in all regions analyzed compared to the stress group in cortex (62,5 %, $p<0.05$), hippocampus (70,58 %, $p<0.05$) and striatum (38,46 %, $p<0.05$). IpE *per se* had no effect on the density of damaged cells. Surprisingly, CGA *per se* caused some cell damage in the brain regions (225 % above control in cortex, 215 % above control in hippocampus and 150 % above control in striatum, $p<0.05$). In contrast, when stressed animals received CGA as pretreatment, there was a partial protection (Figures 4 A (25 %, $p<0.05$), B (52,94 %, $p<0.05$) and C (46,15 %, $p<0.05$) compared to CRS group. Supporting visual evidence for these observations is shown in the photomicrographs of the three brain regions (Cx, Hp and St), presenting the H&E stained tissues in Figure 5 A, B and C.

4. Discussion

In this study we have evaluated for the first time the effects of yerba mate extract, and its major compound CGA, on restraint stress-induced behavioral and morphological alterations in rats. Our results demonstrate that CRS produces brain damage affecting specific brain regions, and compromising important functions of the CNS.

It has been shown that chronic stress is associated with the deregulation of energy supplies, leading to body weight loss in rodents (Gamaro et al., 2003; Flak et al., 2011). This was confirmed in our experiments since CRS was responsible for a significant weight loss, while yerba mate was able to limit this alteration. Although the precise mechanisms by which this protective effect occurs remain unsolved, it is known that stress causes oxidative damage by disrupting the natural balance between pro-oxidant and antioxidant reactions (Zafir et al., 2009). Together with the already reported alterations in energetic metabolism, stress leads to ROS formation, including superoxide radical originated from the mitochondrial electron transport chain (Du et al., 2009). Thus, the antioxidant and protective nature of IpE could be related with its content in polyphenols, which in turn may stabilize the redox status and improve systemic responses to stress, thus helping to preserve major physiological markers such as body weight. However, we cannot discard other possible mechanisms involved in this effect, such as the regulation of the stress response to corticosterone and further hormonal modulation.

Regarding the vulnerability of the brain regions studied to stress, this susceptibility could be related with selective regional alterations in redox status, as these regions are major oxygen consumers, and in general terms, the whole brain (Halliwell and Gutteride, 2007). This could help to explain the morphological alterations found in the striatum, hippocampus and cortex as restraint stress makes neuronal cells vulnerable to

the attack of ROS mainly because neuronal cell membranes are rich in polyunsaturated fatty acids (PUFA) (Friedman et al., 2010), which in turn are major substrates for oxidation. It is known that alterations of cellular homeostasis resulting from enhanced ROS levels or carbonyl compounds can cause modifications on cellular components, particularly proteins (Ambrożewicz and Bielawska, 2016). In turn, damage to nerve cells through these mechanisms along different brain regions (striatum, cortex and hippocampus) might affect motor and cognitive skills, thus representing a risk to compromise major physiological functions.

Natural antioxidants such as polyphenols control ROS generation, the oxidation of proteins and lipid peroxidation (Chen et al., 2012). Our results suggest that cells from the striatum, cortex and hippocampus responded effectively to the stimulus provided by IpE to display antioxidant defense mechanisms. Moreover, other studies have demonstrated that yerba mate possess neuroprotective properties, including antidepressant- (Ludka et al., 2016), anxiolytic- and stimulant-like (Santos et al., 2015) effects, also attenuating dyskinesia and memory dysfunction (Colpo et al., 2007; Costa et al., 2015), as well as reduction of the frequency of pentylenetetrazol-induced seizures (Branco et al., 2013). Herein, we demonstrated once again the anxiolytic potential of IpE. Altogether, these effects emphasize the therapeutic potential of this natural product.

In turn, CGA has been reported as a free radical and metal scavenger, and constitutes the major component of IpE (Colpo et al., 2016). CGA has been shown to improve spatial learning and memory (Han et al., 2010), inducing anti-amnesic activity by reducing of acetylcholinesterase activity and malondialdehyde (MDA) in the hippocampus and frontal cortex (Kwon et al., 2010). CGA has also been involved in the reduction of brain and blood-brain barrier (BBB) damages and brain edema by radical scavenging activity and inhibitory effects on metalloproteinases (Lee et al., 2012).

Although in the present report CGA showed moderate protective effects compared with IpE, the protective potential of CGA against CRS is substantial, not only in consideration to the previous reports aforementioned, but also on the basis of recent experimental evidence collected by us about the protective effects of CGA against the CRS-induced oxidative damage (Colpo et al., 2017). We therefore hypothesize that the protective effects exerted by IpE in this study obey to a synergistic action of CGA with other polyphenols. Several mechanisms, including free radical scavenging, metal chelation and modulation of enzyme activities, have been proposed to explain the protective effects of polyphenols in the brain (Schaffer et al., 2012).

In general terms, evidence support beneficial effects of antioxidants in chronic stress and in redox alterations in neurodegenerative disorders, including Alzheimer's (AD) and Parkinson's (PD) diseases. Zaidi and Banu (2004) demonstrated the protective effects of vitamin E by restoring antioxidant systems in the brain of animals submitted to restraint stress. In the hippocampus, the organosulfur compound found in aged garlic extracts S-allyl cysteine (SAC), exerted a modulatory action on antioxidant responses in acute restraint stress (Colín-González et al., 2015). Hong et al. (2014) showed that Rooibos tea reduces lipid peroxidation, restores stress-induced protein degradation, and modulates GSH metabolism. Our results are in agreement with all these reports, considering that the beneficial effects of IpE and CGA can be due to their antioxidant properties as polyphenols (Leopoldini et al., 2011). Further studies on chronic stress are needed to support our findings and demonstrate specific mechanisms of action of both IpE and CGA.

5. Concluding remarks

IpE produced a protective effect on behavioral and morphological parameters in stressed rats, whereas CGA did not. It is suggested that the large number of antioxidant compounds found in the extract act synergistically in the modulation of these effects. Our results provide evidence that IpE can exert protective effects in the nervous system, besides other known effects. This evidence serve to design and initiate additional experimental paradigms that will help to consolidate the promising properties of this herb, also suggesting that in the future, natural extracts can be used to design treatments for counteracting alterations related with stress in the Central Nervous System.

Ethical approval

All procedures with animals were carried out strictly according to the guidelines established by the Guide for Care and Use of Laboratory Animals NOM-062-ZOO 1999 published by the National Institutes of Health, and following the recommendations of the Ministry of Health of Mexico.

Competing interest

The authors declare that they have no conflict of interest.

Authors' contributions

ME de Lima, AC Colpo, M Maya-López, H Becerríl-Chávez, J Villeda-Hernández and S Galván-Arzate, all contributed to project development, data collection and analysis. ME de Lima and A Santamaría contributed to writing of the manuscript. ME de Lima, V Folmer and A Santamaría designed the whole project. All authors have approved the final manuscript.

Acknowledgements

This work was supported by CAPES (Grant No. 99999.006894/2015-01, MEL), UNIPAMPA (MEL, Brazil) and CONACyT (Grant No. 205648, AS, Mexico).

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Figure captions

Figure 1. Body weight changes (delta) in rats exposed to chronic restraint stress (6 h per day for 21 days) that received (or not) *Ilex paraguariensis* extract (IpE, 200 mg/kg, p.o.) or chlorogenic acid (CGA, 2 mg/mL, p.o.). Mean values ± S.E.M of n = 6 rats per group are shown. $^{+}p<0.05$, different of control (C) group; $^{*}p<0.05$, different of stress (S) group; one-way ANOVA followed by Tukey's test.

Figure 2. Effects of *Ilex paraguariensis* extract (IpE, 200 mg/kg, p.o.) or chlorogenic acid (CGA, 2 mg/mL p.o.) on the restraint stress-induced anxiety-like behavior in rats. A: Number of entries to close arms. B: Time in seconds remaining in close arms. Mean values ± S.E.M of n = 6 rats per group are shown. $^{+}p<0.05$, different of control (C) group; $^{*}p<0.05$, different of stress (S) group; one-way ANOVA followed by Tukey's test.

Figure 3. Effects of *Ilex paraguariensis* extract (IpE, 200 mg/kg, p.o.) or chlorogenic acid (CGA, 2 mg/mL, p.o.) on the restraint stress-induced changes in locomotor activity (vertical and horizontal distance, and total movements) in rats. Mean values ± S.E.M of n = 6 rats per group are shown. $^{+}p<0.05$, different of control (C) group; $^{*}p<0.05$, different of stress (S) group; one-way ANOVA followed by Tukey's test.

Figure 4. Effects of *Ilex paraguariensis* extract (IpE, 200 mg/kg, p.o.) or chlorogenic acid (CGA, 2mg/mL p.o.) on the restraint stress-induced ratio of cell damage in cortex (A) hippocampus (B) and striatum (C) of rats. Mean values ± S.E.M of n = 6 rats per group are shown. $^{+}p<0.05$, different of control (C) group; $^{*}p<0.05$, different of stress (S) group; one-way ANOVA followed by Tukey's test.

Figure 5. Effects of *Ilex paraguariensis* extract (IpE, 200 mg/kg, p.o.) or chlorogenic acid (CGA, 2mg/mL p.o.) on the restraint stress-induced cell damage in cortex (A) hippocampus (B) and striatum (C) of rats. Photomicrographs of the three brain regions (Cx, Hp and St) depicting H&E stained tissues are shown. Representative photomicrographs of all groups are shown contrasting healthy (arrows) vs. damaged cells (arrowheads). Magnification 40x.

Figures

Figure 1

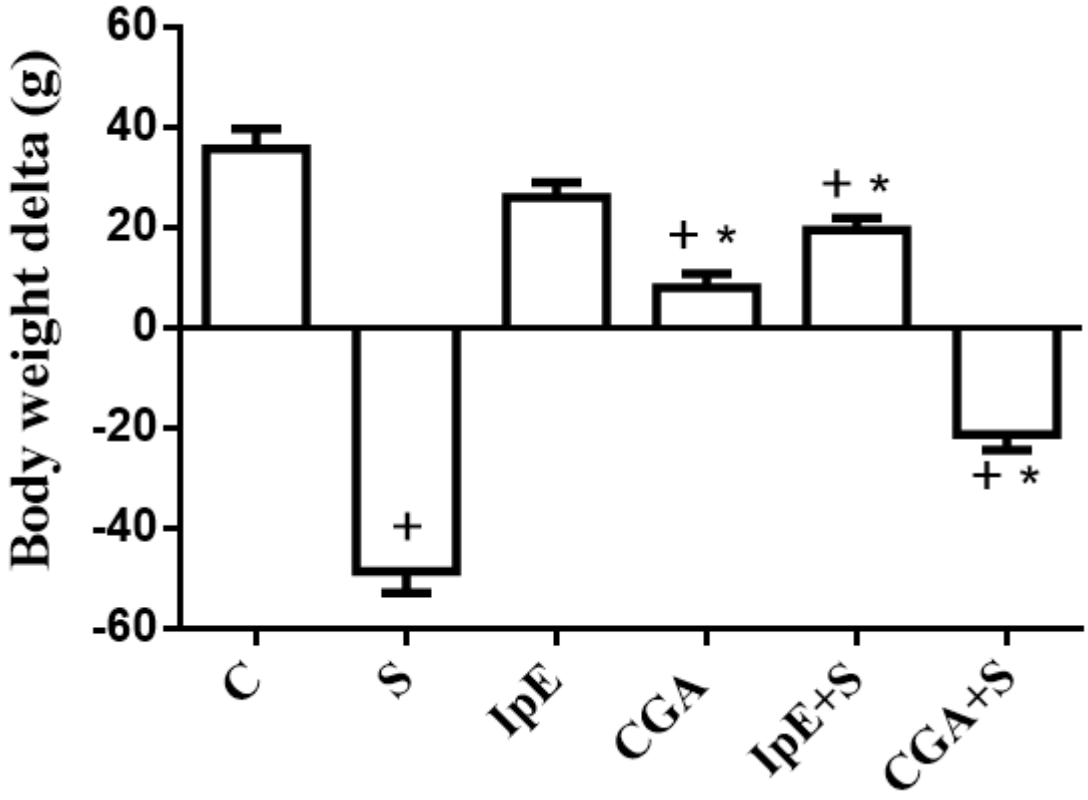


Figure 2

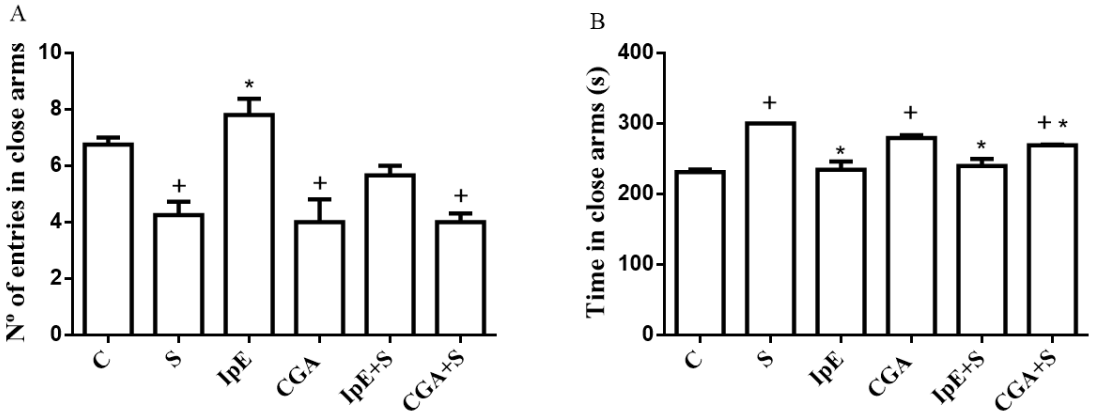
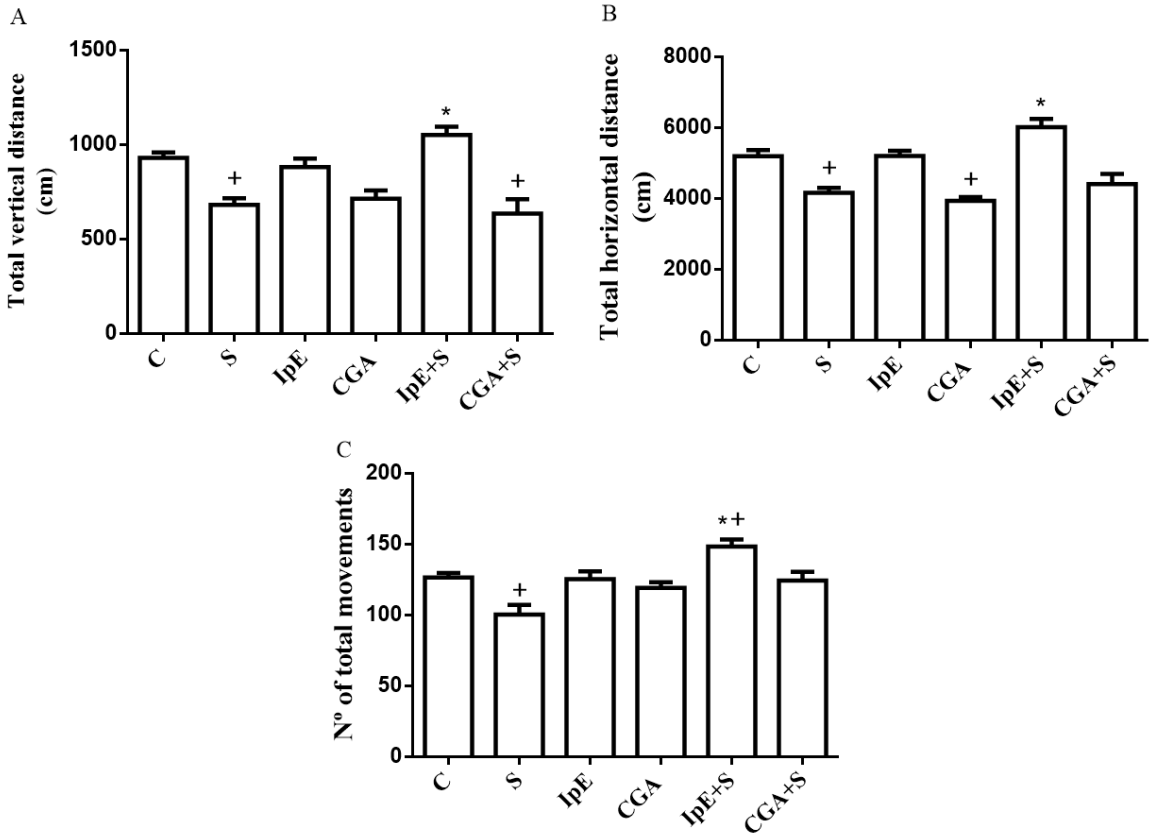

A
Nº of entries in close arms
10
8
6
4
2
0
C
S
IpE
CGA
IpE+S
CGA+S
B
Time in close arms (s)
400
300
200
100
0
C
S
IpE
CGA
IpE+S
CGA+S

Figure 3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IpE
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CGA+S
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6000
4000
2000
0
C
S
IpE
CGA
IpE+S
CGA+S
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S
IpE
CGA
IpE+S
CGA+S

Figure 4

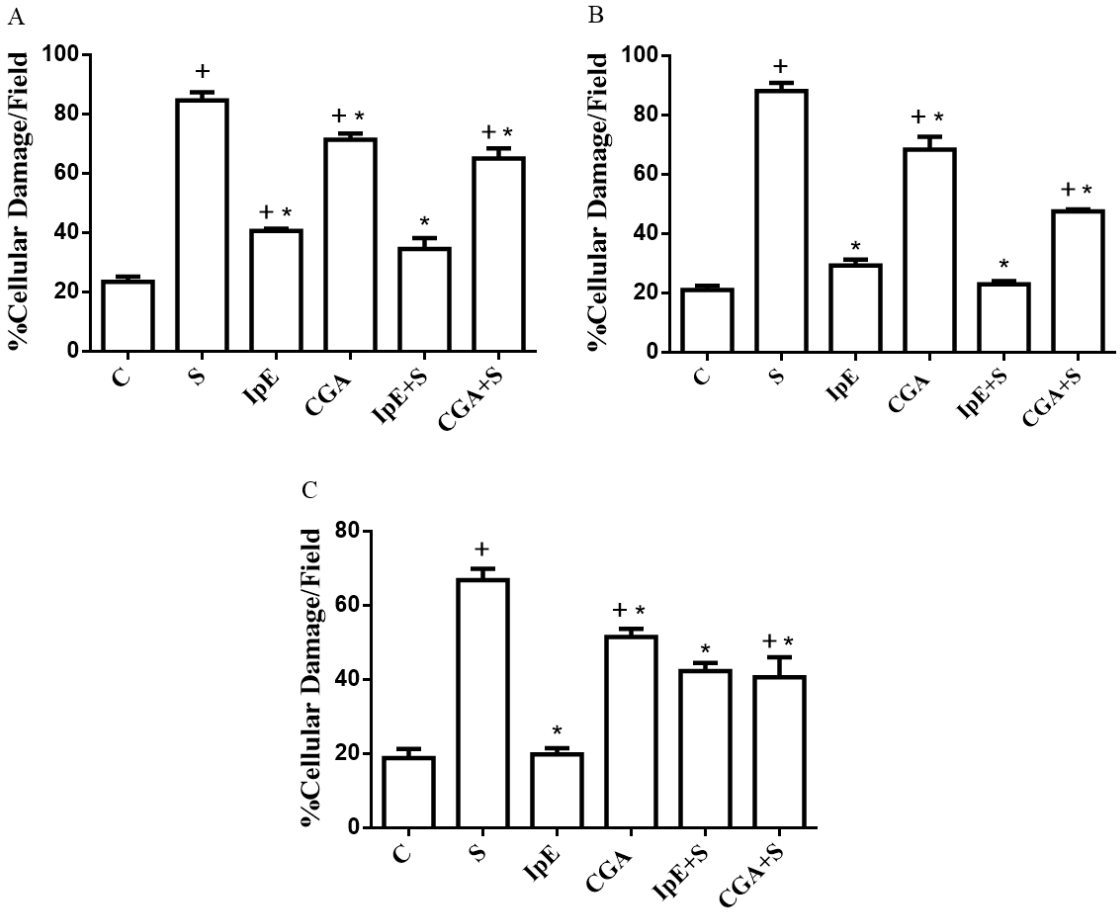
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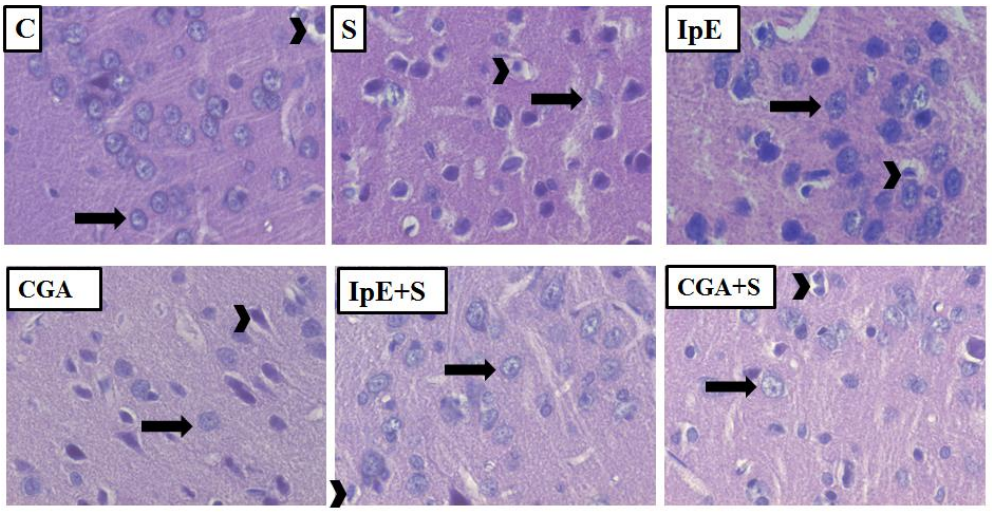
Figure 5

A



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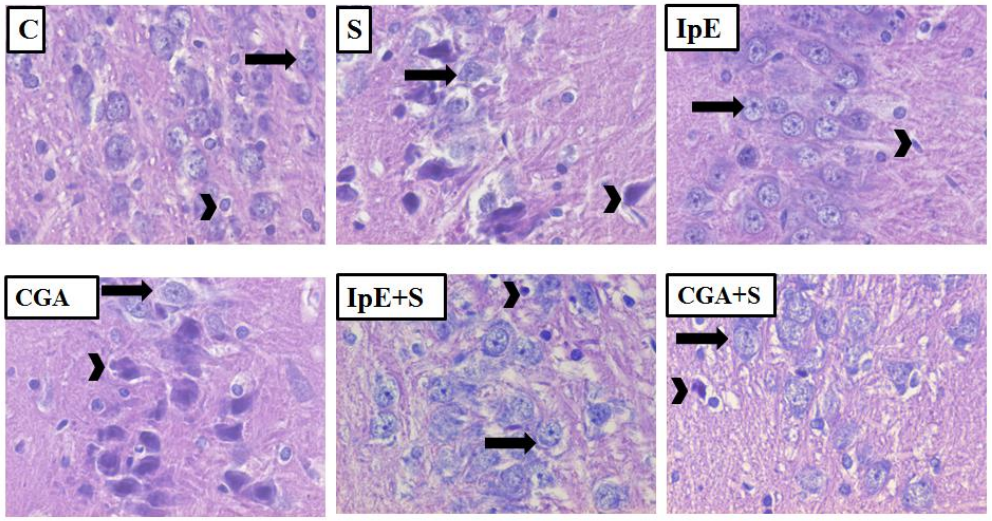

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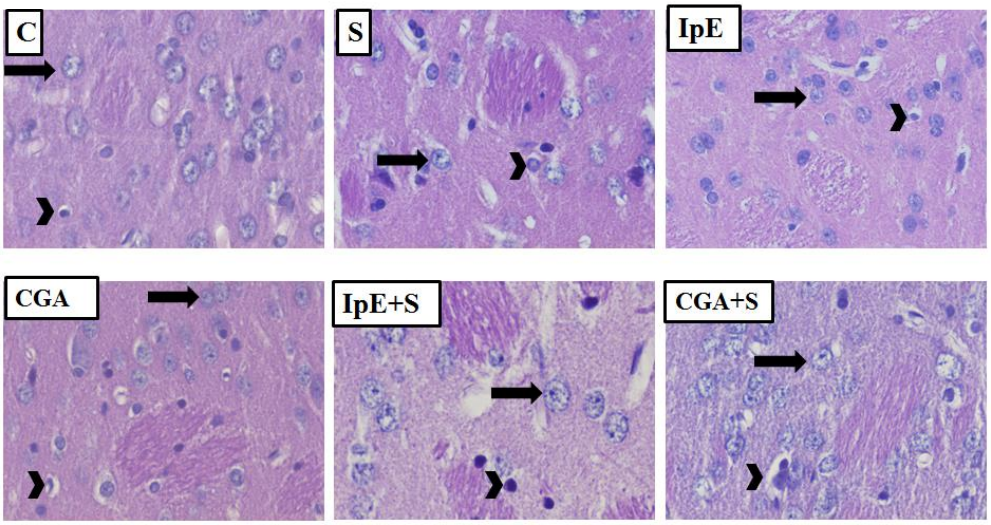

3.3 Manuscrito Científico 2

Manuscrito submetido à

Journal of Functional Foods, quails A2 na CBII.

Elsevier Editorial System(tm) for Journal of Functional Foods

Manuscript Draft

Manuscript Number: JFF-D-17-01882

Title: Ilex paraguariensis extracts reduce blood glucose, peripheral neuropathy and oxidative damage in mice exposed to streptozotocin

Article Type: Full Length Article

Keywords: Ilex paraguariensis; Diabetes mellitus; Oxidative damage; Peripheral neuropathy; Streptozotocin.

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Abstract: Ilex paraguariensis is used for the preparation of stimulant drinks commonly consumed in South America. Extracts of this plant are known to contain a wide variety of polyphenols, exerting antioxidant effects. The aim of this study was to evaluate for the first time the effect of yerba mate extracts in a model of type 1 diabetes induced by streptozotocin (STZ) in mice, considering glucose levels and oxidative stress parameters.We evaluated glucose levels, serum transaminases, and fructosamine in blood and oxidative stress parameters in liver, kidney and brain. Finally, we analyzed the thermal sensitivity as an indicator of diabetic neuropathy. Treatment of mice with Ilex paraguariensis extracts reversed hyperglycemia caused by STZ, normalized oxidative stress parameters and reversed peripheral neuropathy. Our extracts were able to reverse these alterations, and these effects can be attributed to their potent antioxidant capacity combined with a potential hypoglycemic effect thereof.

Ilex paraguariensis extracts reduce blood glucose, peripheral neuropathy and oxidative damage in mice exposed to streptozotocin

Running head: **Streptozotocin, diabetes and Yerba mate**

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This study was supported by CAPES, CNPq and UNIPAMPA.

ABSTRACT

Ilex paraguariensis is used for the preparation of stimulant drinks commonly consumed in South America. Extracts of this plant are known to contain a wide variety of polyphenols, exerting antioxidant effects. The aim of this study was to evaluate for the first time the effect of yerba mate extracts in a model of type 1 diabetes induced by streptozotocin (STZ) in mice, considering glucose levels and oxidative stress parameters.We evaluated glucose levels, serum transaminases, and fructosamine in blood and oxidative stress parameters in liver, kidney and brain. Finally, we analyzed the thermal sensitivity as an indicator of diabetic neuropathy. Treatment of mice with Ilex paraguariensis extracts reversed hyperglycemia caused by STZ, normalized oxidative stress parameters and reversed peripheral neuropathy. Our extracts were able to reverse these alterations, and these effects can be attributed to their potent antioxidant capacity combined with a potential hypoglycemic effect thereof.

Keywords: *Ilex paraguariensis*; *Diabetes mellitus*; Oxidative damage; Peripheral neuropathy; Streptozotocin.

1. Introduction

The search for natural products possessing beneficial biological activity to human health has grown up lately. Among the reasons for this are the high cost of the drugs and its wide range of side effects (Bernardi et al., 2011). In this regard, extracts of the plant *Ilex paraguariensis* (IP) have been shown to exert various effects on a number of pathological events, such as obesity, hypolipidemia and hypoglycemia (Arçari et al., 2009; Pereira et al., 2012).

Chronic hyperglycemia is a clinical condition caused by a metabolic disorder known as *Diabetes mellitus*. Currently, the disease is considered an important public health problem in many countries (IDF, 2015). Thus, a variety of crude extracts and compounds isolated from plants have been evaluated in experimental tests in order to identify new bioactive compounds exhibiting potential beneficial effects for the treatment of this disease (Luceford and Gugliucci, 2005; Zanatta et al., 2007; Cazarolli et al., 2008; Folador et al., 2010).

The evolution of science has enabled several human diseases to be reproduced by experimental models in laboratory animals. These models allow us to test potential treatments that in the future can be extrapolated to human use. Streptozotocin (STZ) is a glucosamine-nitrosourea commonly used to produce diabetes (Silva et al., 2011). STZ is a toxin obtained from *Streptomyces achromogenes* which, being synthesized by yeast, has very similar structure to glucose molecule, and therefore, its uptake is facilitated into pancreatic cells that contain the GLUT-2 glucose transporter (Elsner et al., 2000, Delfino et al., 2002).

After being incorporated by cells, STZ develops its cytotoxic mechanisms, leading to death of pancreatic β-cells and the consequent failure in the production and release of

the hormone insulin, therefore leading to the development of chronic hyperglycemia and featuring the metabolic disorder *Diabetes mellitus* type 1 (Silva et al., 2011).

In addition to hyperglycemia, the damaging processes caused by STZ recruit the formation and release of reactive oxygen species (ROS). Thus, animals treated with STZ develop most of diabetic complications (such as diabetic neuropathy (Comelli et al., 2009) and body weight loss (Wang et al., 2010)) through oxidative stress.

Previous studies in our group have demonstrated the potential antioxidant effects of *Ilex paraguariensis* extracts, as well as its ability to increase lifespan in animal models by reducing oxidative damage caused by external toxins (Colpo, 2016; Lima et al, 2014). Considering the increased incidence of diabetes in human populations, and the need to seek for alternative therapies, in this work we evaluated the effects of IP extracts in an animal model of type 1 diabetes induced by STZ, estimating the glucose levels in blood, peripheral neuropathy and oxidative stress parameters in mice. Our results show protective actions of IP extracts on these endpoints of toxicity.

2. Materials and Methods

2.1. Chemicals

Ellman's reagent (DTNB) and STZ (reagent grade) were obtained from Sigma Aldrich (St. Louis, MO, USA); commercial kits were supplied by Labtest (Minas Gerais, Brazil). Other reagents were obtained from local suppliers.

2.2. Yerba mate samples

According to results previously described by our group (data not shown), Uruguayan IP extracts showed better phytochemical composition and antioxidant activity, compared to other commercial herbs, including Argentinian and Brazilian. Therefore, we chose to use the Uruguayan herb in the present study (Colpo et al., 2016).

The commercial presentation of yerba mate was acquired in the municipal Uruguayan market of Bella Unión. The traceability of the herb was ensured by strict legislation and rules that regulate the production of herbs in Brazil.

2.3. Obtaining IP extracts

Aqueous extracts were obtained by recreating the traditional *mate* preparation process, according to Lima et al. (2014). *Mate* was prepared in a medium size gourd, with the amount of yerba mate occupying two thirds of the volume of the bowl (60 g). Free volume was completed successively with water (70 ml) at 80°C. Water in the bowl remained in contact with yerba mate for one minute. Then, the water was taken out through a pump attached to a suction system (described below). The extracts were obtained successively (one after the other), and the infusions (1, 2, 5, 10, and, 15) were stored. The 15th infusion is equivalent to the 1 L amount of water usually consumed by people taking mate. The time of extraction was one minute. The other extractions were discarded.

The *mate* pump was attached by means of a rubber hose to a Kitasato flask. The bottle cap allows the connection because it is composed of a silicone stopper with a hole which passes through a glass tube. The extract passes through the system and falls into the Kitasato flask, which is connected to a vacuum pump that is responsible for the suction (based on the method described by Meinhart et al., 2010). Afterwards, the extracts were filtered using a paper filter, thickness 205 µm (J.Prolab®, S.J. dos Pinhais, Brazil), and stored in falcon tubes. After the extraction, the material was stored and kept frozen in a freezer (-18°C) to administration by gavage.

2.4. Instrumentation and chromatographic analysis

The chromatographic system consisted of a Prominence liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a CBM- 20A controller, LC-20AT pump, SIL 20a auto sampler and SPDM20A DAD detector. The chromatographic separation was achieved using an ODS-Hypersil Thermo Scientific C18 column (250 x 4.6 mm i.d., 5 µm particle size) at 25 ± 2 °C. The mobile phase consisted of water containing acetic acid (0.3%, solvent A) and methanol (solvent B). The gradient elution started with 15 – 20% B for 20 min; 20 – 85% B for 5 min, and maintained it for more 5 min (Filip et al., 2001). The flow rate was 1.0 ml/min and injection volume was 20 µl. Data acquisition by the PDA system was monitored at 265 nm for caffeine and theobromine, and at 325 nm for caffeic and chlorogenic acids. Before analyses, the mobile phase was filtered through a 0.45 µm membrane filter in a solvent filtration apparatus (Millipore, USA) and sonicated. The reference standards (chlorogenic acid, caffeic acid, theobromine and caffeine) were purchased from Sigma–Aldrich® (St. Louis, MO, USA) and prepared as 50% hydroethanolic solvents. Different wavelengths were considered at 325 nm for phenolic derivatives and 265 nm for methylxanthines. All samples, including mates, were filtered through 0.45 mm nylon filters and injected into the HPLC equipment.

2.5. Animals

All experiments were performed using 2-3 month-old male Swiss mice (25-35 g). A total at 32 animals were housed in groups at a controlled room temperature (22 ± 2ºC) and humidity (60-80 %), under a 12-12 h light-dark cycle (lights on at 07:00 a.m.), and with food and water available *ad libitum*. All experimental procedures were based on the ‘‘Principles of Laboratory Animal Care’’ from the NIH publication No. 85-23, the International Standards of Animals recommended by the Brazilian Law (#11.794 - 10/08/2008), and approved by the Animal Ethics Committee of the Fundação Universidade Federal do Pampa.

2.6. Diabetes induction and IP treatment

STZ was prepared in fresh citrate buffer (0.05 M, pH 4.5) and administered to animals after 4 hours of fasting at a dose of 100 mg/kg, i.p., according to Salgueiro et al. (2016). Five days after STZ administration, hyperglycemia was confirmed using ACCU-Check Active (Roche Diagnostics). IP treatment (850 mg/kg by gavage) started after confirmation of hyperglycemia and lasted for 12 weeks. The animals were distributed into four groups:

- G1: Control untreated: received only citrate buffer i.p. and drank water throughout the whole period;
- G2: STZ untreated: received a single STZ dose (100 mg/kg, i.p.) and drank water throughout the whole period;
- G3: Control Mate: received only citrate buffer i.p., drank water *ad libitum* and IP extracts by gavage once a day.
- G4: STZ + Mate: received a single STZ dose (100 mg/kg, i.p.), drank water *ad libitum* and IP extracts by gavage once a day.

2.7. Assessment of response to thermal stimulation by hot plate

The response to thermal stimuli was evaluated before and after induction of diabetes, once a week during 12 weeks of treatment. This response was tested on a hot plate equipment, according to previous reports (Goecks, 2011).

2.8. Evaluation of glycemic profile

Prior to euthanasia, glucose blood levels were measured in blood sample obtained from the tail of mice, using a glucometer.

2.9. Tissue preparation for biochemical analyses

After exposed to treatments, animals were sacrificed and whole blood was collected in heparinized tubes by cardiac puncture. The liver, kidneys and brain were removed and homogenized in cold NaCl (0.9 %). The homogenates were centrifuged at 4,000 g for 10 min at 4º C, and the supernatants were then collected for biochemical analyses.

2.10. Evaluation of serum fructosamine levels, and AST and ALT markers

The levels of fructosamine and liver damage markers (AST and ALT) were evaluated in blood samples using commercial kits (Labtest, Minas Gerais/Brazil).

2.11. Measurement of SOD, CAT, δ-ALA-D enzyme activities

The activity of superoxide dismutase (SOD) was determined according to the protocol proposed by Kostyuk and Potapovitch (1989). Catalase (CAT) activity was estimated according to the protocol proposed by Aebi (1984). The activity of δ-ALA-D was assessed by measuring the formation of porphobilinogen (PBG), in accordance with the method described by Sassa (1982).

2.12. Evaluation of lipid peroxidation

The levels of lipid peroxidation in tissues were determined as the levels of TBA reactive substances (TBA-RS), according to the method proposed by Ohkawa et al. (1979).

2.13. Analysis of the levels of non-protein thiols (SHNP)

The levels of non-protein thiols were assessed according to the method described by Ellman (1959), and modified by Maldonado et al. (2006). All analyses were corrected by protein content, using Bradford's method (Bradford, 1976).

2.14. Statistical analysis

Graphic data were expressed as mean ± SEM. Statistical evaluation of the results was carried out using a two-way analysis of variance followed by *post hoc* Tukey's test. Data were analyzed using GraphPad Prism 6.0 software (San Diego, CA, USA). Statistical significance was reached at $p<0.05$ values.

3. Results

3.1 Phytochemical composition of Ilex paraguariensis extract

Chromatographic analysis showed four marker components present in IP mate (data not shown); these phenolics and methylxanthines have been identified as theobromine (6.5 min), chlorogenic acid (9.43 min), caffeic acid (18.42 min) and caffeine (18.42 min), according to their retention time and UV absorbance compared to purified standards. According to the standard peak-area ratio, the order of relative amounts of the compounds found in mate was as follows: chlorogenic acid (11.82 mg/g) > caffeine (8.26 mg/ g) > caffeic acid (7.44 mg/g) > theobromine (5.07 mg/g), respectively.

3.2 Ilex paraguariensis extract reduce glucose levels, glycosylated blood proteins and normalize the level of hepatic enzyme AST in plasma

The results obtained in this study confirm the efficacy of the experimental model since 100 mg/kg STZ caused an increase in blood glucose levels (Fig. 1A) and glycosylated blood proteins (Fig. 1B), and altered liver function (Fig. 1C and 1D). These data allowed us to verify that, after three months of treatment with yerba mate extracts, glucose levels and glycosylated proteins in the blood in the STZ + Mate were normalized almost to baseline levels (Figs. 1A and 1B). Besides that, after treatment with IP extracts, we observed a partial improvement of hepatic function in mice evidenced by blood levels of liver enzyme AST, but not in ALT (Fig. 1C and 1D).

3.3 Ilex paraguariensis extract reduce lipid peroxidation in liver, kidney and brain

As seen in Figure 2, STZ increased lipid peroxidation in mice liver (Fig. 2A), kidney (Fig. 2B) and brain (Fig. 2C), expressed herein as the levels of TBA-RS. The treatment with IP extract was able to reduce lipid peroxidation in the three tissues analyzed by decreasing the level of reactive species close to the level of control group.

3.4 Ilex paraguariensis extract normalizes the redox status of tissue evaluated by the modulation of activity of the enzymes SOD and CAT

Experimental model of diabetes showed increased activity of the antioxidant enzymes superoxide dismutase (SOD) (Figure 3) in liver (Fig. 3A) and kidney (Fig. 3B), as well as catalase (CAT) in liver (Fig. 3C), kidney (Fig. 3D) and brain (Fig. 3E). Treatment with IP extract normalized the activity of SOD in liver and kidney, as well as the activity of CAT in liver and brain compared to control levels.

3.5 Ilex paraguariensis extract normalized non-protein thiols (NPSH) levels in liver, kidney and brain.

The STZ-induced diabetes model caused increased levels of non-protein thiols (Figure 4) in mice liver (4A), kidney (4B) and brain (4C). After 12 weeks of treatment with IP extract, the levels of NPSH were normalized to control level in liver, kidney and brain tissues.

3.6 Treatment with Ilex paraguariensis prevented the accumulation of ALA substrate in the liver

Streptozotocin caused an inhibition in the activity of the ALA-D enzyme in liver, kidney and brain (Figure 5), which produced a subsequent accumulation of ALA substrate in these tissues. IP extract normalized the liver (Fig. 5A) ALA-D activity after treatment, but not in the kidney (Fig. 5B) and brain (Fig. 5C).

3.7 Ilex paraguariensis extract reduces peripheral neuropathy in diabetic animals

Diabetic mice presented signs correlated with peripheral neuropathy, evidenced as a longer time of contact of the paws in a hot plate. Treatment with IP extract significantly reduced the response time of animals to this test (Figure 6).

4. Discussion

In the present study we evaluated whether the IP extracts could be benefitial in the reduction of glycemia and re-establishment of the redox status of some tissues. In fact, some hypoglycemic effects of the extracts were found, reducing the glycemia and the level of glycosylated proteins in the plasma of STZ-treated mice. This result can be explained by the phytochemical composition of the extract, which, as shown, is rich in phenolic compounds, mainly chlorogenic acid. Altogether, these IP components have been described to exert a wide range of biological and pharmacological activities (Pereira et al, 2012; Lima et al, 2014).

The capacity of C-glycosylated flavonoids to exert effects on insulin secretagogue has been described in the literature (Cazarolli et al., 2009; Folador et al., 2010). However, in our model, after the destruction of pancreatic B cells, no insulin production occurred. Therefore, we hypothesize that IP extracts might exert insulin-like effects, favoring the intracellular uptake of glucose, thereby reducing blood glucose and the levels of glycosylated proteins associated with the levels of fructosamine. Broadhurst et al. (2000) found similar results when analyzing and comparing forty nine herbs, spices, and herbal extracts in an insulin-dependent model. These authors found an insulin-like effect in some herbs analyzed and concluded that the polyphenols present in the extracts were responsible for this effect.

Aminotransferase enzymes are widely distributed in the body and are present in high concentrations in hepatocytes. Necrosis, or any disorder that causes hepatic damage, increases blood levels of intracellular enzymes such as AST and ALT (Scott et al., 1984;. Wang et al., 2010; Xu et al., 2014). In the experimental model of STZ-induced diabetes, we found an increase in AST and ALT plasma levels. After treatment with IP extracts, we observed a partial improvement of hepatic function in mice evidenced by

blood levels of liver enzyme AST. Thus, we suggest that our extracts exerted partial protective activity in liver in this model. Similarly, Janbaz et al. (2003) found a protective effect of caffeic acid and quercetin under elevated transaminase levels in blood following contact with a substance that produces hepatic toxicity. These authors corroborated our explanation that the properties of these compounds represent a contributing factor in this effect.

On the other hand, the mechanism of tissue damage taking place in our diabetes model appears to be common to all injured cells, and results as a consequence of cellular oxidative stress after increased ROS production, which may be due to auto-oxidation of glucose, formation of advanced glycation end products (AGEs), and alterations in the content and activity of the whole antioxidant defense system in tissue (Reis et al., 2008; Silva et al., 2011). It is also believed that the oxidation of lipids and proteins is associated with the development of complications in this syndrome (Rosen et al., 2001; Folmer et al., 2002; Brownlee, 2005).

The hyperglycemia observed in mice treated with STZ may lead to the formation of hydrogen peroxide, which in turn generates free radicals such as O_2•- and OH•. These reactive species cause lipid peroxidation resulting from the formation of endoperoxides and fatty acid hydroperoxides, which produce increased malondialdehyde formation and thromboxane-B2 (Negri, 2005).

IP components have been described to exert a wide range of biological and pharmacological activities (Pereira et al, 2012; Lima et al, 2014). Protective effects of the tested extracts can be attributed to their antioxidant properties linked to their composition, therefore exerting protective actions on lipid peroxidation in the STZ-treated animals receiving IP extracts. Our findings are in agreement with previous reports describing this effect (Venkateswaran and Pari, 2003; Negri, 2005)

Since during oxidative stress there is an increased generation of ROS, the endogenous defense systems sense this condition and increase their activity as a compensatory response (Halliwell and Gutteridge, 2007), as evidenced in this study with SOD and CAT activities, and NPSH levels. In contrast, the tested extracts acted normalizing the levels of CAT and SOD enzymes, as well as the levels of non-protein thiols. We suggest that these effects are due to the phytochemical composition of the extracts, whose main component is chlorogenic acid, a well-known antioxidant. Phenolic compounds are considered as exogenous antioxidants acquired through the diet (Sousa et al., 2007). When there is a supplementation with yerba mate extracts, it is likely that their components acted neutralizing excess ROS induced by diabetes, thus normalizing the enzymatic activity of SOD and CAT, and non-protein thiol levels.

Herein, we also show that STZ inhibited the activity of δ-ALA-D enzyme in all analysed tissues. This results in the accumulation of 5-aminolevulinic acid (ALA). ALA, under pathological conditions, acts as pro-oxidant (Smith et al, 2012), thus aggravating the cellular oxidative damage caused by hyperglycemia (Pereira et al., 1992; Bechara et al., 1993; Folmer et al., 2003). Our results show that treatment with IP extracts prevented the damage caused by STZ. These treatments protected against the inhibition of ALA-D enzyme in mice liver, but not in kidney or brain, improving the redox status of hepatic cells. Performing similar analyzes, Smith et al. (2012) found that green tea reversed the inhibition of δ-ALA-D enzyme in mice, and attributed this effect to the total content of polyphenols found in tea. Derived from this conclusion, we suggest that the effects shown in this study exerted by the IP extracts are due to their bioactive compounds acting as exogenous antioxidants, thus contributing to cell protection and reversing the damage caused by STZ in mice.

Diabetic neuropathy is the most common complication of diabetes mellitus, and is considered the main factor of morbidity and mortality in this disease (Basić-Kes et al., 2011). Since the STZ model used herein can mimic diabetic neuropathy, we used the hot plate behavioral test to evaluate thermal sensitivity. We observed damage to the nervous system evidenced by peripheral neuropathy caused by STZ. Animals receiving STZ spent more time with the feet in contact with the hot plate than control mice, indicating loss of peripheral sensitivity caused by neuropathy. In contrast, animals that received yerba mate extracts spent less time in contact with the plate, indicating that peripheral sensitivity was restored to the level of control mice.

The pathogenesis of peripheral neuropathy has been studied in several animal models. However, several groups report no reproducibility of the changes caused by this condition in humans (Cameron et al., 2001; Obrosova et al., 2009). Among the several possible mechanisms linked to this condition are non-enzymatic glycation and glyco-oxidation of biomolecules (Cameron et al., 2005; Toth et al., 2008), activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) (Price et al 2004; Cheng et al., 2010), and oxidative and nitrosative stress (Cameron et al., 2001; Coppey et al., 2000; Obrosova et al., 2004). Here we demonstrate that treatment of mice with yerba mate extracts reduced peripheral neuropathy induced by STZ. We therefore hypothesize that this effect was produced by the bioactive compounds present in IP; among these are phenolic compounds known for their powerful antioxidant activity (Sousa et al., 2007). Macro and microvascular complications of diabetes mellitus also affect the nervous tissue (Philippe et al., 2014). About 60 % of all diabetics develop peripheral neuropathy related with alterations in thermal and mechanical sensitivity (Vincent et al., 2011).

In summary, the extracts tested in this study exhibited a variety of beneficial effects, reversing the damage caused by STZ in mice most probably through their well-known antioxidant properties.

5. Conclusion

In the present report, we confirmed the efficacy of the model of type 1 diabetes in mice induced by STZ, and demonstrated the protective effects of IP extracts on oxidative stress parameters, blood glucose levels, liver damage markers and peripheral neuropathy. The IP extracts tested here demonstrated efficacy to reverse the complications caused by chronic hyperglycemia in the animal model of type 1 diabetes. These findings, together with evidence collected from the literature, suggest that IP compounds may help for the design of therapeutic alternatives for the treatment of diabetes mellitus by reducing oxidative stress and other damaging mechanisms in this disease.

Competing interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by CAPES and UNIPAMPA.

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Figures captions

Figure 1. Glucose, fructosamine, AST and ALT levels in mice blood. + Indicates differences vs. control (untreated), and *indicates differences vs. STZ group. Data were analyzed by two-way ANOVA followed by *post hoc* Tukey's ($p<0.05$).

Figure 2. Levels of lipid peroxidation (TBA-RS formation) in mice liver (Fig.2A), kidney (Fig. 2B) and brain (Fig. 2C). + Indicates differences vs. control (untreated), and *indicates differences vs. STZ group. Data were analyzed by two-way ANOVA followed by *post hoc* Tukey's ($p<0.05$).

Figure 3. SOD activity in mice liver (Fig. 3A) and kidney (Fig. 3B); and CAT activity in mice liver (Fig. 3C), kidney (Fig. 3D) and brain (Fig. 3E). + Indicates differences vs. control (untreated), and *indicates differences vs. STZ group. Data were analyzed by two-way ANOVA followed by *post hoc* Tukey's ($p<0.05$).

Figure 4. NPSH levels in mice liver (Fig.4A), kidney (Fig. 4B) and brain (Fig. 4C). + Indicates differences vs. control (untreated), and *indicates differences vs. STZ group. Data were analyzed by two-way ANOVA followed by *post hoc* Tukey's ($p<0.05$).

Figure 5. δ-ALA-D activity in mice liver (Fig.5A), kidney (Fig. 5B) and brain (Fig. 5C). + Indicates differences vs. control (untreated), and *indicates differences vs. STZ group. Data were analyzed by two-way ANOVA followed by *post hoc* Tukey's ($p<0.05$).

Figure 6. Evaluation of thermal sensitivity in mice by hot plate. + Indicates differences vs. control (untreated), and *indicates differences vs. STZ group. Data were analyzed by two-way ANOVA followed by *post hoc* Tukey's ($p<0.05$).

Figures

Figure 1

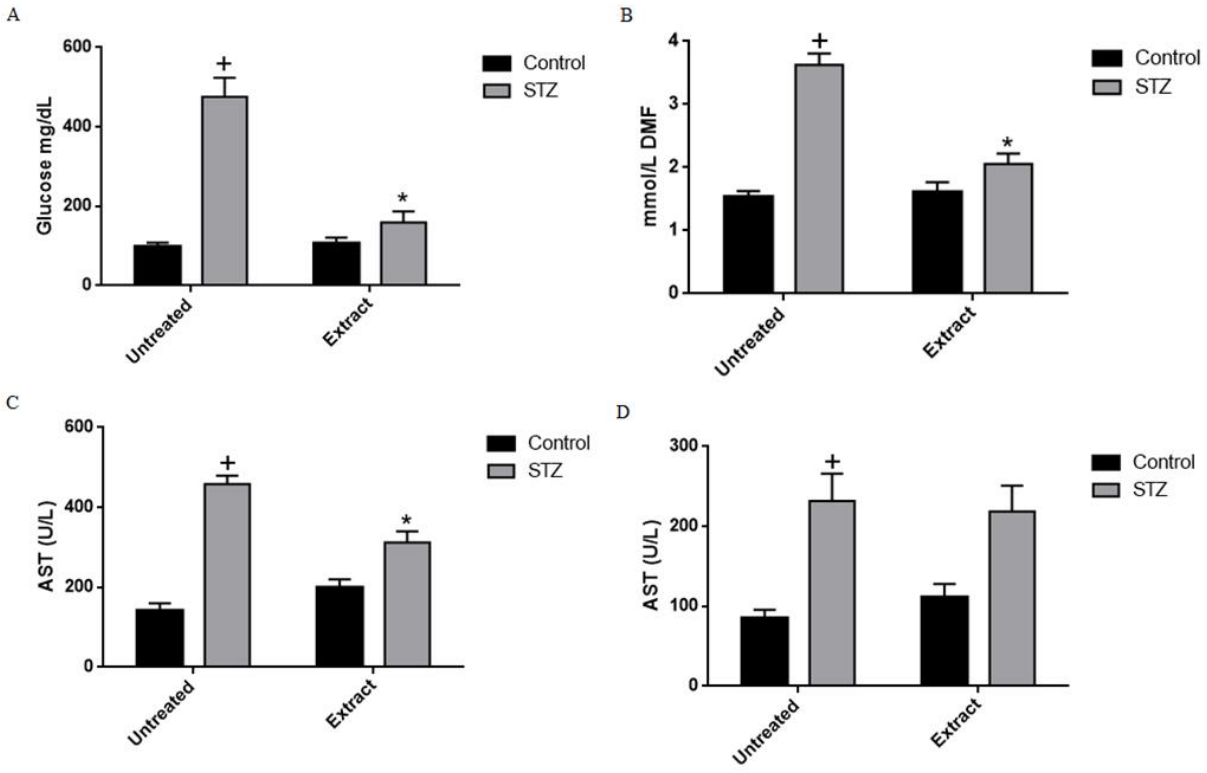

Figure 2

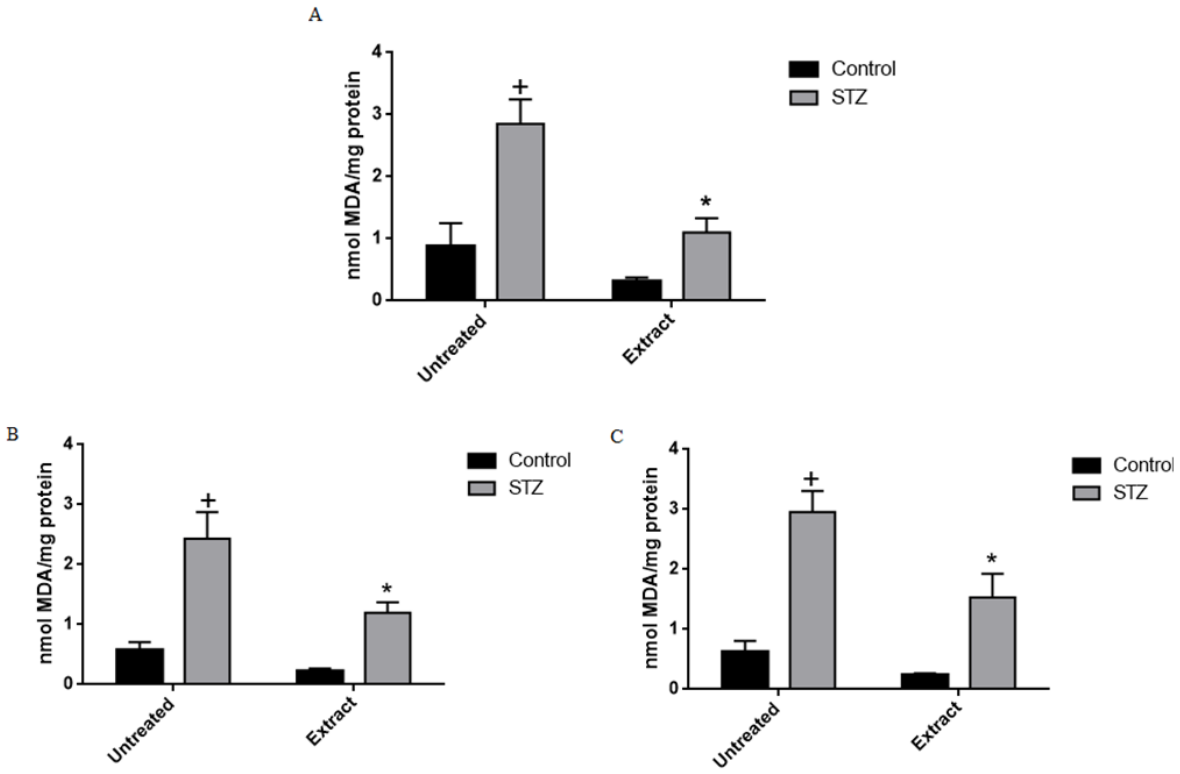
A
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Figure 3

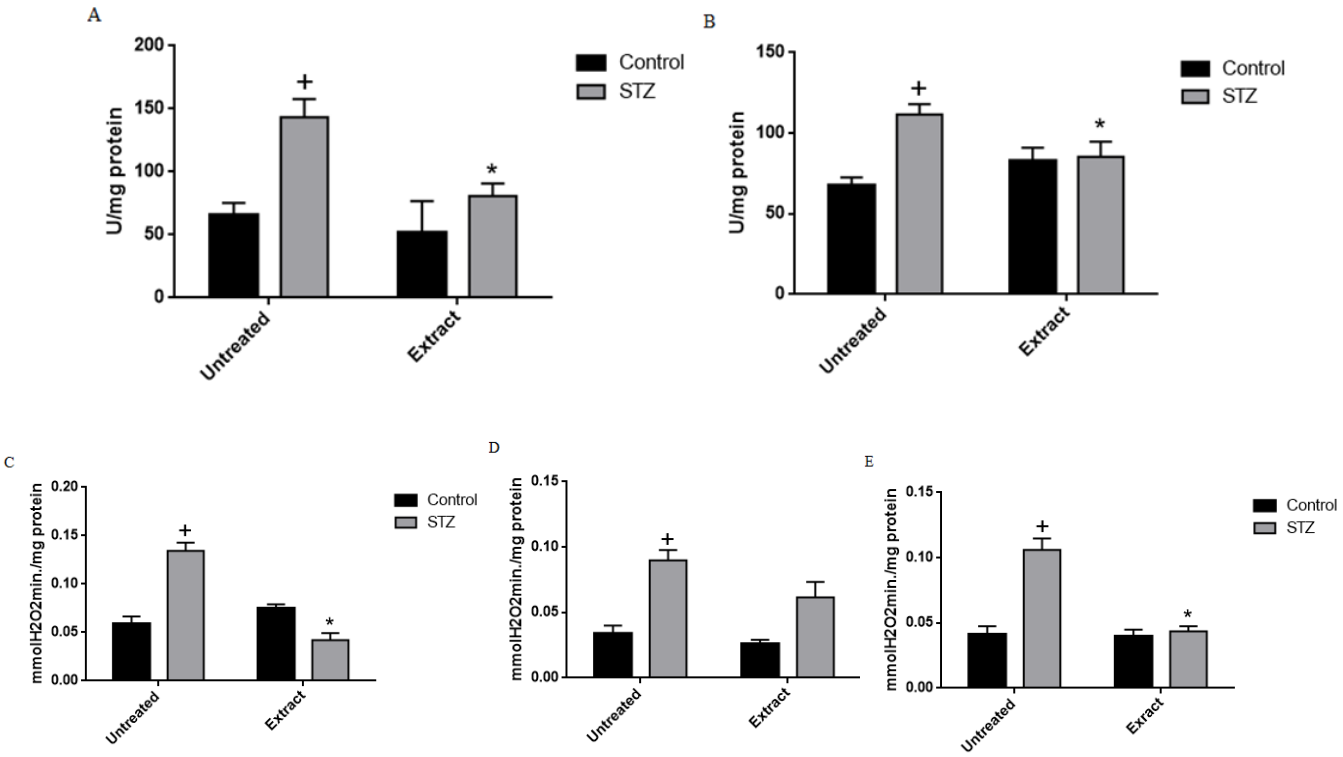
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Figure 4

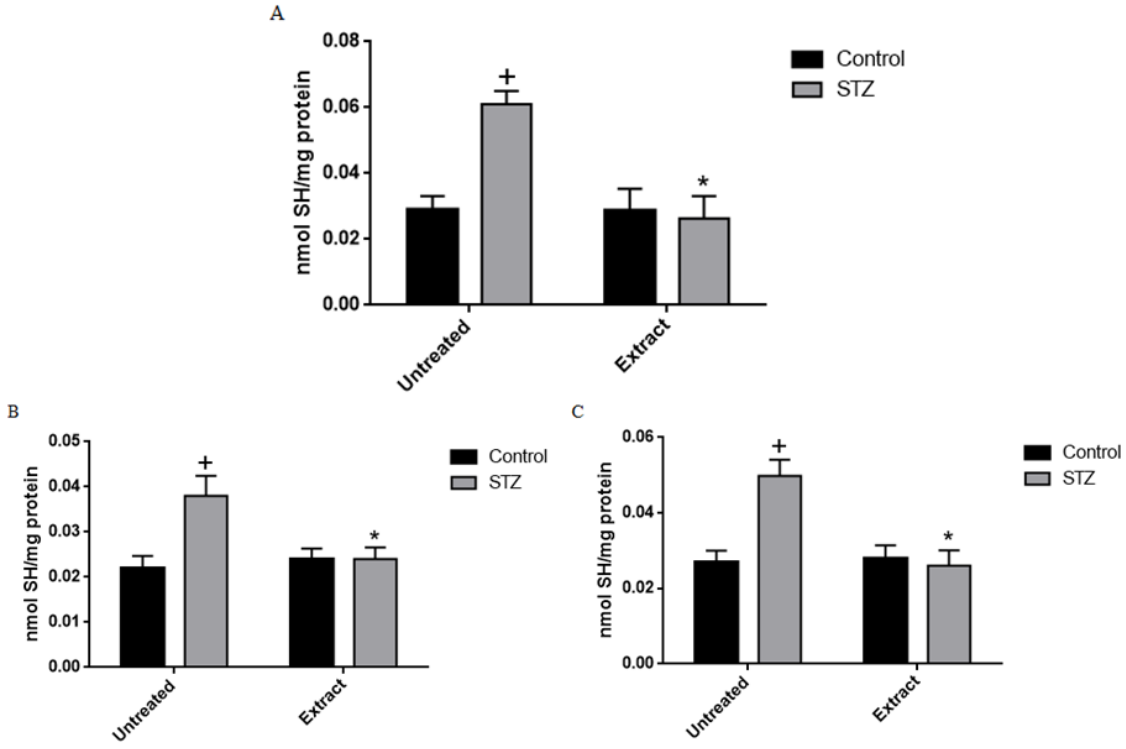
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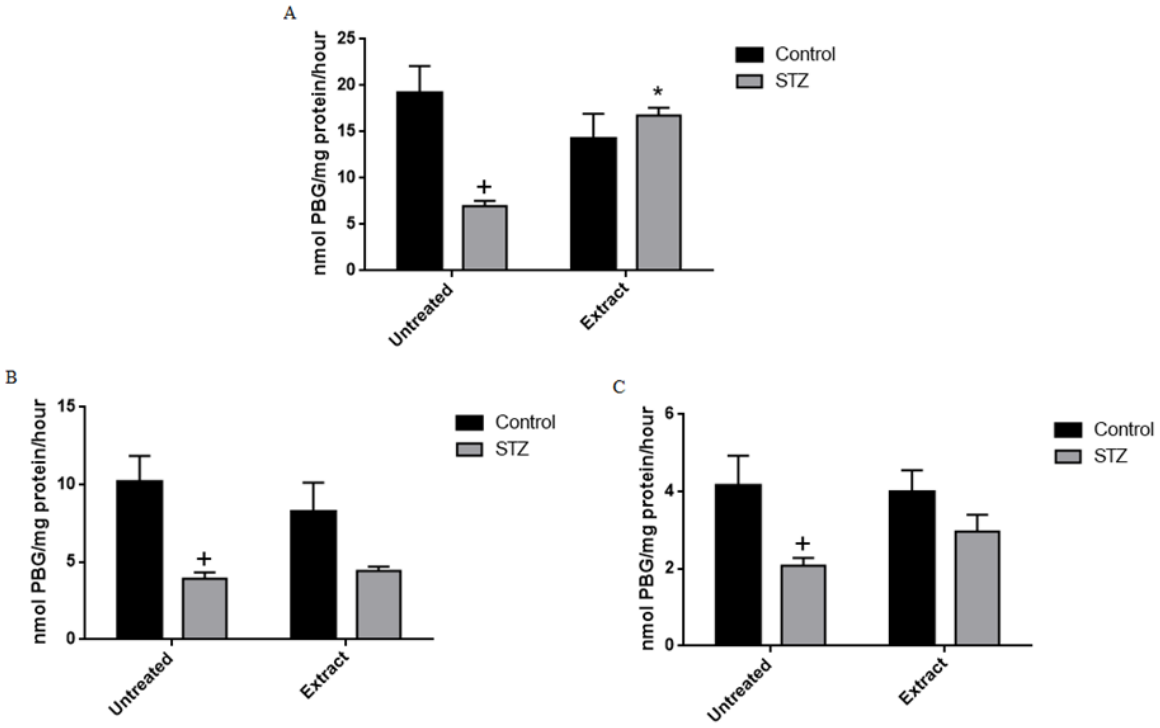
Figure 5


A
nmol PBG/mg protein/hour
25
20
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10
5
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STZ
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B
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15
10
5
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Control
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C
nmol PBG/mg protein/hour
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2
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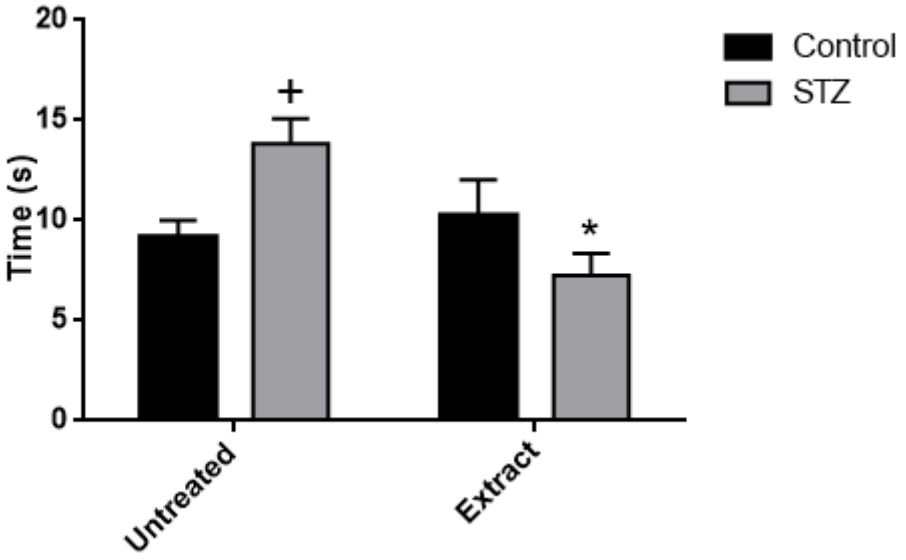
Untreated
Extract

Figure 6



4 DISCUSSÃO GERAL

No presente trabalho exploramos pela primeira vez os efeitos benéficos e protetores dos extratos de *Ilex paraguariensis* (Ip) em modelos biológicos *in vivo* e *in vitro* a partir de diferentes condições patológicas.

Inicialmente, pensando-se em avaliar a unidade funcional e estrutural de comunicação do SNC, as sinapses, utilizamos terminações nervosas isoladas do cérebro de ratos na presença de alguns agentes tóxicos. Adicionalmente, também comparamos os efeitos dos extratos de *Ilex paraguariensis* com os do ácido clorogênico, em marcadores bioquímicos de dano como a função mitocondrial, dano oxidativo a lipídios e produção de ROS, bem como a taxa de glutationa reduzida/oxidada como um índice de defesa antioxidante dessas estruturas. Os extratos de *Ilex paraguariensis* tiveram um efeito protetor ao dano oxidativo induzido em sinaptossomas, através da redução da produção de ROS , redução da peroxidação de lipídios e também preveniram a depleção de glutationa, o que refletiu diretamente na proteção da função mitocondrial exibida.

Orientando a investigação para avaliação *in vivo* no SNC, utilizamos um mecanismo de lesão indireta e avaliamos marcadores gerais funcionais e estruturais, comparando novamente os efeitos da erva mate com o seu componente majoritário, o ácido clorogênico (Colpo et al., 2016; Lima et al., 2017).

O modelo de estresse por restrição de movimentos afeta a motricidade, governada pela função córtico estriatal e também a ansiedade, controlada pela função córtico hipocampal dos animais. Tais efeitos comportamentais são correlacionados com algum grau de deterioração morfológica das três regiões envolvidas (Colín-González et al., 2015; Becerríl-Chavez et al., 2017).

Os animais estressados, que não receberam tratamento, indicam uma possível redução do caráter exploratório, naturalmente exibido por roedores, além de um comportamento

ansiolítico elevado, corroborando com dados previamente encontrados (Becerril-Chávez et al., 2017).

O estresse por restrição de movimentos gera dano oxidativo podendo inclusive, causar a morte celular em diferentes regiões cerebrais, conforme já relatado por alguns autores (Radak et al., 2001; Colín-González et al., 2015; Becerril-Chávez et al., 2017). De fato, nossos dados mostraram uma correlação entre as alterações comportamentais e um incremento no dano celular de córtex, hipocampo e estriado *ex vivo*.

Os efeitos do extrato de erva mate foram superiores aos do ácido clorogênico tanto nos parâmetros comportamentais, quanto à citotoxicidade. Os animais apresentaram inclusive uma maior atividade locomotora, em comparação com o controle. Este fato pode ser explicado devido ao extrato possuir na sua composição cafeína (Colpo et al., 2016), conhecida como estimulante do SNC (Alves et al.,2009).

Os dados encontrados apontam evidências de que há um sinergismo dos compostos bioativos dos extratos de erva mate, pois quando isolou-se o composto fenólico principal, ácido clorogênico, este apresentou atividade inferior.

Recentemente, nosso grupo publicou um artigo que apoia nossos resultados e considerações (Colpo et al., 2017), em tal reporte, foi demonstrado que no mesmo modelo de estresse crônico, o extrato de erva mate preveniu diferentes marcadores de estresse oxidativo, dando a este evento o caráter causal de dano ao Sistema Nervoso Central e justificando os efeitos protetores dos compostos testados.

Por fim, pensamos em estimar o efeito da erva em nível geral, sem envolver mecanismos específicos, através da avaliação de aspectos dos sistemas antioxidantes e da vulnerabilidade dos diferentes órgãos frente ao dano produzido por um modelo mimético de diabetes tipo 1.

De fato, o modelo de indução com estreptozotocina, conforme já relatado na literatura (Furman et al., 2015), provoca destruição das células beta pancreáticas, com consequente aumento da glicemia dos animais devido a deficiência de insulina.

Na diabetes, a hiperglicemia crônica é considerada o fator de risco para o desenvolvimento das complicações pela doença (Tiwari & Rao, 2002). A primeira consequência da hiperglicemia crônica é a produção aumentada de ROS (Reis, 2008), o que leva a ativação de diferentes vias metabólicas, conforme pode ser visto na figura 5. Por conseguinte, os eventos que seguem a ativação das vias culminam com a situação de estresse oxidativo, que ocorre pela ação de redução dos sistemas antioxidantes endógenos (Santini et al., 1997; Wajchenberg, 2002; Reis, 2008).

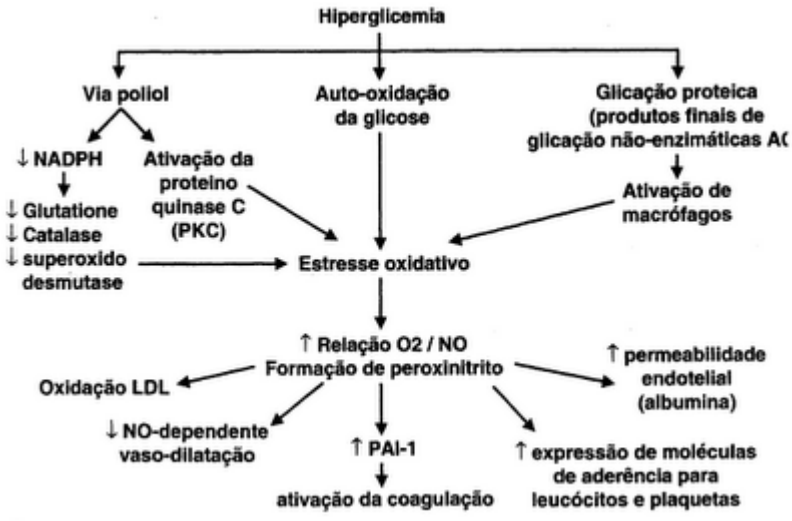


Figura 5 – Associação da ativação das vias metabólicas e o estresse oxidativo no diabetes.
Fonte: Wajchenberg, 2002.

Schmatz et al. (2012) observaram que ratos diabéticos apresentaram, após 30 dias, uma redução na atividade das enzimas antioxidantes, bem como no nível de tióis não proteicos, o que foi prevenido nos animais que foram tratados com resveratrol. Contrariamente, os nossos resultados apresentaram um aumento na atividade das enzimas e também no nível de tióis não proteicos em fígado, rim e cérebro dos camundongos diabéticos.

O tratamento com extrato de erva mate durou 90 dias, desta maneira, acreditamos que os efeitos encontrados tenham sido uma resposta compensatória do organismo dos animais frente ao dano causado pela estreptozotocina, o que corrobora com os dados de Salgueiro et al., 2016.

O extrato de erva mate foi capaz de normalizar as defesas antioxidantes ao nível do controle e também prevenir a neuropatia diabética nos animais tratados. A neuropatia diabética está diretamente relacionada com o estresse oxidativo resultante do aumento do fluxo de glicose pela via dos polióis. Na referida via, o sorbitol é convertido a frutose, o que leva a um aumento na formação de seus derivados, frutose-3-fosfato e 3- deoxiglicosonas, que são agentes da glicação não enzimáticas (AGEs) (Chung et al., 2003;Rocha et al., 2006; Reis, 2008).

Os AGEs se acumulam na maioria dos órgãos-alvo que podem ser acometidos no diabetes, e além disso, tem sido relatados por agregarem-se aos nervos periféricos, piorando assim a neuropatia diabética devido à redução da velocidade de condução nervosa e do fluxo sanguíneo para o local (Hammes et al., 1999; Chen et al., 2004; Barbosa et al., 2008).

Acredita-se que os efeitos relatados do extrato de *Ilex paraguariensis* no modelo de diabetes induzido por estreptozotocina sejam devido às propriedades antioxidantes já relatadas (Oliveira et al., 2011;) através de diferentes mecanismos, desde a sua capacidade em capturar espécies reativas de oxigênio (ROS) (Olthof et al., 2001; Lima et al., 2017) , até a sua capacidade moduladora da atividade redox através da modulação da atividade e expressão do sistema de defesa enzimático (Pereira, 2013) que a erva mate apresenta.

Além disso, alguns autores explanam que derivados fenólicos podem possuir atividade inibitória sobre as enzimas chave, como a aldose redutase, α-amilase (Matsuda et al., 1998; Kobayashi et al., 2000), ou também, podem atuar inibindo a absorção de glicose, o que pode explicar a redução da glicemia nos animais (Welsch et al., 1989; Tiwari e Rao, 2002).

Os efeitos protetores dos extratos foram efetivos nos três modelos desenvolvidos através, presumivelmente, das ações que seus diversos compostos exercem, sobretudo antioxidante, frente à exacerbada atividade pró-oxidante apresentada pelas diferentes condições tóxicas testadas.

Em cada modelo, as ações antioxidantes dos extratos de Ip, foram capazes de reduzir ou inibir os diversos marcadores de toxicidade avaliados. Juntamente com os efeitos benéficos exercidos pelo ácido clorogênico, os resultados obtidos com os extratos de Ip sugerem, de maneira sólida um amplo espectro de atividade terapêutica a nível experimental por parte deste composto natural.

As implicações destes resultados impactam não somente a investigação biomédica ao sustentar a eficácia de compostos naturais ricos em antioxidantes contra eventos tóxicos gerados por diversas patologias, se não também o nível social e cultural, ao demonstrar que compostos naturais que são consumidos tradicionalmente por populações específicas constituem um elo da biomedicina moderna com a cultura tradicional.

Portanto, as repercussões do presente trabalho fomentam que infusões de erva mate devem seguir sendo consumidas como hábito cultural pois podem exercer efeitos benéficos à saúde do povo que a consome (Dutra et al., 2010; Bracesco et al., 2011; Júnior & Morand, 2016).

Os compostos fenólicos, especialmente os flavonoides, que constituem, entre outros componentes, a erva mate, possuem uma ampla atividade antioxidante (Pietta, 2000; Havsteen, 2002; Denisov & Afanas’ev, 2005), atuando na redução do estresse oxidativo e podendo exercer atividade benéfica em outras alterações celulares paralelas como a inflamação (Arçari et al., 2011;Puangpraphant et al., 2011), disfunção mitocondrial e déficit energético (Lima et al., 2017), excitotoxicidade (Schinella et al., 2000; Anesini et al., 2012) e a morte celular (Bixby et al., 2005).

Já foi demonstrado que alguns compostos do extrato de erva mate, como ácido clorogênico e a cafeína, atuam no SNC via barreira hematoencefálica na sua forma intacta ou através de metabólitos (Ito. et al., 2008), o que explica em parte os efeitos protetores aqui expostos. Além disso, um estudo mais recente (Oliveira et al., 2013) verificou a biodisponibilidade de extratos de erva mate e constatou que os ácidos clorogênicos livres foram os principais ácidos fenólicos presentes no estômago e intestino grosso, enquanto no plasma, fígado e urina foram encontrados metabólitos, especialmente o ácido cafeico ligado ao ácido glicurônico.

Sem embargo, com os resultados aqui apresentados, podemos conferir aos extratos de erva mate efeitos hipoglicemiantes, antiexcitotóxicos, citoprotetores e reguladores da motricidade. A diversidade destes efeitos modula diferentes vias que estes compostos podem estar regulando nos organismos vivos e que devem ser mais exploradas em estudos posteriores.

Uma alternativa para explicar a inibição de processos diversos de dano através da modulação redox radica na regulação de vias de sinalização mediadas pela estimulação de fatores de transcrição de resposta antioxidante, como o Nrf2. Esse fator é conhecido como regulador mestre de respostas antioxidantes em organismos vivos (Jaiswal, 2004; Singh et al., 2010; Johnson & Johnson, 2015), e tem um reportado cruzamento com vias inflamatórias, como NFkB, inibindo as moléculas pró inflamatórias (Linker et al., 2011; Scannevin et al., 2012).Em estudos futuros, avaliaremos o papel deste fator na sinalização antioxidante e o seu efeito em outras vias de dano confluentes.

O denominador comum nos modelos avaliados é o dano oxidativo, e o fator comum nos compostos testados é o seu caráter antioxidante. Desta maneira, propomos a hipótese de que o potencial antioxidante e modulador redox dos extratos de erva mate, bem como a ação do ácido clorogênico, já reportado como antioxidante e indutor da ativação de Nrf2 (Sato et

al., 2011; Jang & Kim et al., 2017), são os responsáveis pela proteção encontradas em todos resultados desta tese.

Conferimos ao estresse oxidativo o valor causal de dano em nível central e periférico em diferentes patologias, e sustentamos o valor dos antioxidantes para o desenho de terapias comuns a estas doenças. Afirmamos então que tanto a diabetes, como as alterações produzidas pelo estresse e as doenças neurodegenerativas dividem o elemento comum de dano oxidativo e o potencial de ser moduladas por antioxidantes (Rocha et al., 2006; Salgueiro et al., 2016; Colpo et al, 2017; Lima et al., 2017)

Adicionalmente, um papel realmente neuroprotetor dos flavonoides encontrados nos extratos de Ip requer uma caracterização experimental mais detalhada, dado que a citoproteção demonstrada em nossos estudos não implica neuroproteção de maneira específica. A este respeito, futuros estudos avaliando a capacidade neuroprotetora específica dos flavonoides dos extratos de Ip deve ser realizada através da avaliação da experessão de proteínas neuronais específicas, tais como NeuN e MAP2, as quais podem ser encontradas como parte do citoplasma do citoesqueleto dos neurônios (Mullen et al, 1992; Riederer et al., 1995). A perspectiva de encontrar propriedades neuroprotetoras para estes compostos através de diferentes modelos experimentais de neurotoxicidade constitui uma alternativa *per se* para continuar nossos estudos.

Em resumo, os resultados prometedores das nossas investigações não só sustentam efeitos protetores de componentes ativos de *Ilex paraguariensis,* mas também fomentam uma investigação mais profunda dos mecanismos que esses agentes exercem para gerar tal proteção em modelos tóxicos diversos.

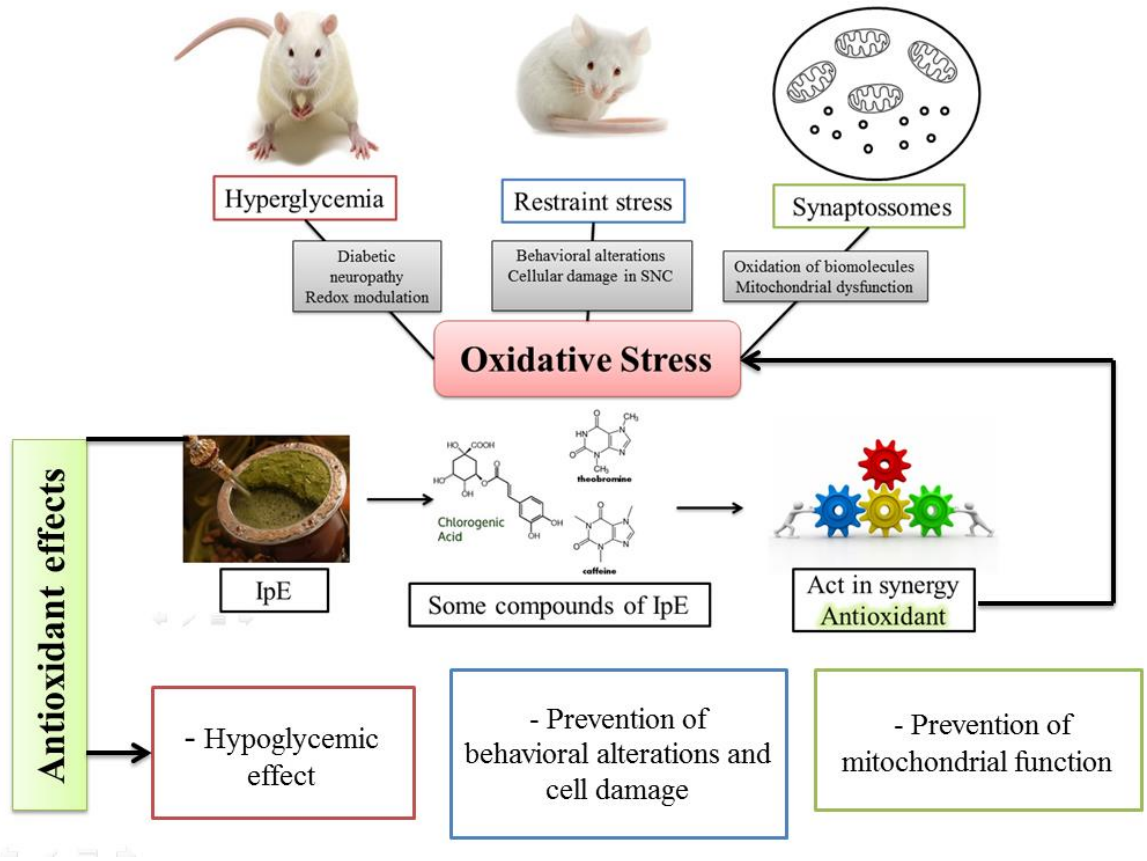


Figura 6 – Efeito sugerido para *Ilex paraguariensis*.

5 CONCLUSÕES

De acordo com os resultados apresentados nesta tese pode-se concluir que os extratos de erva mate apresentam efeito hipoglicemiante e preventivo da neuropatia diabética bem como redução do nível do marcador de dano hepático AST e os níveis de frutosamina em camundongos diabéticos induzidos com estreptozotocina,

Além disso, o extrato também apresentou efeito benéfico *ex vivo* na modulação redox de alguns marcadores antioxidantes como a normalização da atividade das enzimas superóxido dismutase, catalase, delta aminolevulinato desidratase. O extrato normalizou ainda o nível de espécies reativas ao ácido tiobarbitúrico e de tióis não proteicos, nos animais diabéticos.

Com relação ao modelo de estresse por restrição de movimentos, o extrato foi mais efetivo que o ácido clorogênico, pois melhorou a atividade locomotora e reduziu a atividade ansiolítica dos ratos estressados. Ademais, o extrato protegeu hipocampo, córtex cerebral e corpo estriado *ex vivo* do dano celular induzido pelo estresse, nesses animais.

Por conseguinte, o extrato de erva mate exibiu novamente efeito superior ao do ácido clorogênico na proteção ao dano oxidativo induzido *in vitro*, em sinaptossomas. Verificamos uma redução da formação de ROS, menor peroxidação de lipídios e manutenção da razão GSH/GSSG nos níveis adequados, o que deve ter influenciado na proteção da função mitocondrial observada neste modelo.

O conjunto de dados encontrados aponta que os extratos de erva mate atuam de maneira sinérgica reduzindo o estresse oxidativo e dessa maneira, atenuando o dano de algumas condições clínicas que cursam com estresse na sua patogênese.

6 PERSPECTIVAS

A partir dos resultados apresentados nesta tese, têm-se como perspectiva o desenvolvimento de estudos que verifiquem os mecanismos envolvidos nos efeitos dos extratos de erva mate aqui expostos.

Estudaremos os mecanismos específicos envolvidos nos padrões de proteção da erva mate, através de estudos de sinalização e regulação/ modulação de vias. Temos um interesse particular na possível ativação das vias antioxidantes como a Nrf2/Keap1/ARE e o possível silenciamento ou modulação de vias inflamatórias como a NFkB/IkBa/IRE. Acompanhado desses estudos, abordaremos os padrões de atividade e expressão de proteínas antioxidantes e pró/anti-inflamatórias.

Com estas novas aproximações, daremos aos efeitos de erva mate uma nova dimensão de ações a níveis celular, bioquímico e molecular.

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